STUDIES ON THE HYDROPHOBIC AND HYDROPHILIC PROPERTIES OF HUMIC SUBSTANCES

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Chemistry

at the

University of Canterbury

by

Raewyn M. Town

1991

No experiment is ever a complete failure inasmuch as a well written account of it can serve admirably as a bad example.

ACKNOWLEDGEMENTS

I sincerely thank my supervisor and friend Dr H. Kipton J. Powell. His encouragement, support and guidance have contributed greatly to this work. Kip, I appreciate your dedication and skilled teaching. Merci bien mon cher ami.

I extend my gratitude to (Grandad) Dr T. Mark (Fark) Florence (CSIRO, Lucas Heights, Sydney) for facilitating my period of study leave at Lucas Heights ("Seedney, 'Stralia"). Mark's advice and considerable expertise have made a significant contribution to the algal assay studies.

I especially thank Ms Jenny L. Stauber (CSIRO, Lucas Heights, Sydney) for extensive assistance with the algal assays and the radiochemical work.

I am grateful to Dr Graeme E. Batley (CSIRO, Lucas Heights, Sydney) for many helpful discussions on stripping anodically.

I thank Dr John J. Fardy (ANSTO, Lucas Heights, Sydney) for assistance with the radiochemical studies and for providing a copy of the program SIAS ("See-ass").

I thank Dr Jan E. Gregor (DSIR, Ilam Research Centre, Christchurch) for many helpful discussions and for performing the Superquad analyses.

The University Grants Committee is thanked for the award of a Post-Graduate Scholarship.

ABSTRACT

This thesis presents a study on the solubility and aggregation properties of humic and fulvic acids, and their interaction with hydrophobic metal complexes and with aqueous metal ions. A parallel study involved quantitative analysis of solution equilibria between Al(III), Cu(II) and simple ligand systems representing possible complexing moieties in humic substances.

The solubility and aggregation properties of humic acid were studied as a function of pH and ionic strength by gel permeation chromatography and equilibrium dialysis. Predominantly smaller molecules were dissolved below pH 4; the solubility of the larger humic molecules increased with increased pH and with decreased ionic strength.

The potential of XAD resins for the isolation of humic acid from soil extracts was investigated. These macroporous adsorbents were found to be unsuitable for this purpose. Large molecules were excluded from the XAD resins; those components which were adsorbed could not be completely desorbed by strongly alkaline solutions. In conjunction with the solubility studies these results highlighted the operational nature of the fraction defined as 'humic acid' and raised questions about the reported differences between aquatic and soil derived humic substances.

The interaction of humic substances with hydrophobic Cu(II) complexes (L = 1-(2-pyridylazo)-2-naphthol and 8-hydroxyquinoline) was studied by ASV (at a NCTMFE) and by visible absorption spectroscopy. Humic substances (HS) interacted hydrophilically with these species (if necessary displacing a low molecular weight ligand) to form a ternary complex, HS-Cu-L. Algal assays established that humic acid could thus ameliorate the extreme toxicity of hydrophobic Cu(II) complexes provided that the displaced ligand itself is not toxic.

The apparent lability of Cu(II) and Pb(II) complexes with humic and fulvic acids was studied by anodic stripping voltammetry (ASV). At a hanging mercury drop electrode (HMDE) the Cu(II) complexes of humic and fulvic acids were of similar lability; the apparent lability decreased as the pH increased. Complexes of humic substances with Cu(II) were less labile than those with Pb(II). Results were dependent on the electrode system used for the measurements. The effects of adsorption of humic substances on several electrode surfaces were characterized (HMDE, thin mercury film (TMFE), Nafion-coated TMFE, and bare glassy

carbon). These effects were exerted predominantly in the deposition step. A method for measuring the apparent lability of metal-humic complexes was developed which corrected for the contribution from adsorption.

Ion selective electrode (ISE) potentiometry was used to probe the complexation capacity of humic and fulvic acids for Cu(II) (at pH 5.0, 6.3, and 7.0) and the relative stability of these complexes (pH 2.5 - 7.5). For both humic substances the complexation capacity increased with pH. Assuming bidentate coordination then, for fulvic acid at pH 5.0, 6.3, and 7.0 respectively, complexation capacity measurements indicated that 82 - 85%, 67 - 72%, and 50 - 60% of carboxyl groups were not involved in strong Cu(II) binding under the experimental conditions. For humic acid the proportions were 73 - 79%, 33 - 43% and 5 - 25% respectively. Cu(II) complexes with fulvic acid were significantly less stable than those with humic acid. At the same carboxyl group concentration, Cu(II) binding curves for humic acid were displaced markedly to lower pH, indicating stronger binding. For a 1:20 Cu(II):COOH ratio the pH displacement between the humic and fulvic acid curves was 0.65 at pH 3.5 and 1.0 at pH 5.0. The larger humic acid molecules (fractionated by 0.025 μm filtration in weakly acidic solution) were stronger complexors than were the smaller moieties. The apparent stability of Cu(II)-humic complexes decreased with increased metal-to-ligand ratio and with increased ionic strength. The competition of Mg(II) and Al(III) for humic Cu(II) complexation sites was studied. Simulation of the Cu(II) binding curves for humic substances identified the discrete ligands malonate and citrate as reasonable models for humic chelating sites; complexation by salicylate and phthalate moieties was considerably weaker than that by humic substances.

Quantitative pH potentiometric studies were made on the complexation of Cu(II) and Al(III) by carboxylate ligands (25°C, 0.1 mol L^{-1} KCl). The models which best described the solution equilibria in the Al(III) systems were:

Al(III)-malonic acid: AlL (log K = 6.71), AlL_2 (4.94), AlL_3 (2.61), AlL_2OH (-7.0)

Al(III)-isocitric acid: AlHL (10.07), AlL (6.96)

The Cu(II)-5-methoxy-N-(2-hydroxybenzyl)sarcosine system (CuL (7.05), $CuLH_{-1}$ (-3.98)) demonstrated the contribution of 'cascade binding' to metal complex stability.

Formation of insoluble species in the Cu(II)-butane-1,2,3,4-tetracarboxylic acid system prevented quantitative analysis.

CONTENTS

Page

CHAPTER 7: ANODIC STRIPPING VOLTAMMETRY. STUDIES ON THE APPARENT LABILITY OF Cu(II) AND Pb(II) COMPLEXES WITH HUMIC AND FULVIC ACIDS

CHAPTER 1

INTRODUCTION

Humic substances are a complex polydisperse, heterogeneous mixture of macromolecules which are poorly characterized. They are ubiquitous in soils, sediments, lakes, rivers, and oceans. Humic substances are operationally defined on the basis of solubility: fulvic acid is soluble in both acid and alkali, whereas humic acid is precipitated in acid solution.

1.1 FORMATION OF HUMIC SUBSTANCES

The total soil organic matter is comprised of nonliving components which are predominantly products of microbial and chemical transformations of organic debris. These 'transformations' represent the process of humification which generates 'humic substances'. Humic substances characteristically have some resistance to further microbial degradation. It is important to note that the synthesis and alteration of humic substances is a *dynamic* process (Hayes & Swift, 1978).

The mode of formation of humic substances is the subject of debate. Several mechanisms have been proposed including lignin degradation, polyphenol polymerization, and nonenzymatic polymerization of reducing sugars and amino acids (Maillard reaction) (Stevenson, 1982). Each of these pathways could occur to some extent, with the dominant processes involved being dependent on the environment (soil, freshwater, or marine) and, say, the particular soil type. The Maillard reaction has been the more popular mechanism in recent literature. For, example, Ikan et al. (1986b) considered ^{13}C NMR evidence for heterocyclic and heteroaromatic moieties (specifically, furanoid, hydroxy-alkyl-furanone, and hydroxy-alkyl-cyclopentenone) in humic substances and melanoidins to be consistent with this theory.

It is not known if fulvic acids are precursors for humic acid synthesis (Stevenson, 1982) or if they arise from humic acid degradation (Ertel & Hedges, 1984).

Humic substances often 'interfere' with the application or interpretation of many experimental techniques (e.g. by adsorption on electrode surfaces). The heterogeneity of humic substances means that any measured parameter is an 'average' of the contributions from each fraction, weighted in an unknown way. For this reason, some authors have favoured fractionation into more homogeneous components prior to characterization (Wershaw et al., 1988). Unfortunately, this may cause alteration of the original humic sample. Further, the fractionation processes (e.g. by molecular weight) are operationally defined and are unlikely to give 'true' separations. Many of the attempts to fractionate humic substances have reduced the heterogeneity of the sample but none have isolated materials that could be considered as 'pure' chemicals, or even a homogeneous group of chemicals. "In this regard, humic substances represent a unique category of natural products in which the essence of the material appears to be heterogeneity *per se*" (MacCarthy & Suffet, 1989). Hence, studies on humic substances present some unique difficulties. According to MacCarthy and Suffet (1989), this makes the investigation of humic substances "a particularly challenging and exciting endeavor" (!).

1.2 ENVIRONMENTAL IMPORTANCE OF HUMIC SUBSTANCES

It is desirable to further understand the structure and function of these complex materials and their geochemical reactions. The environmental importance of humic substances is far reaching. The chelation of metal ions by humic substances and their interactions with hydrophobic contaminants has a significant impact on the speciation, immobilization, transport, bioavailability, and biogeochemical cycling of these species in the environment. In addition, humic substances can be both a source and a sink for atmospheric carbon dioxide, they are a buffer against acid precipitation, and they have a significant impact on the fertility and moisture holding capacity of soils (Averett et al., 1989). Humic substances also interact with major nutrients such as ammonia, nitrates, phosphates, and silicates; they enhance soil fertility, terrestrial plant growth, and aquatic productivity (Prakash & MacGregor, 1983). Further, they are reported to have many beneficial medical

applications ranging from analgesic and anticarcinogenic activity to the treatment of stomach and intestinal disorders (Visser, 1988).

Recently, increased mobilization of metal ions into groundwaters as a result of acid precipitation (and the concomitant increase in toxicity to aquatic organisms) has generated interest in the chelation and speciation of metal ions by humic substances (the ligands dominating complexation in natural waters). Due to the extreme toxicity of aluminium to aquatic and terrestrial life, its interaction with humic substances has been of particular interest (Backes & Tipping, 1987; Tipping & Backes, 1988; Tipping et al., 1988a,b; Mulder et al., 1989). In general, free Al(III) and its monomeric hydroxy products are considered to be the most toxic species (Helliwell et al., 1983; Rosseland et al., 1990); humic-bound Al(III) may not be bioavailable (Gunn et al., 1986).

Chlorination has been widely used as a disinfectant for water supplies for many years. Recently, its use has become controversial; chlorination of humic substances generates trihalomethanes which are potential carcinogens. Consequently the products formed on chlorination of humic substances, and their toxicity, is a rapidly developing field of research (El-Rehaili & Weber, 1987; Vartiainen et al., 1987; Italia & Uden, 1988; Jacobs et al., 1988; Kronberg et al., 1988; Agarwal & Neton, 1989; Reckhow et al., 1990).

Humic substances are the major light absorbing compounds in natural waters; therefore they play an important role in photochemical reactions in aqueous systems. The interaction of sunlight with humic substances generates reactive transient species (e.g. H_2O_2) which are involved in redox reactions and may interact with organic contaminants (Zepp et al., 1981a,b; Baxter & Carey, 1982; Haag et al., 1984; Zepp et al., 1985; Faust & Hoigne, 1987; Frimmel et al., 1987; Cooper et al., 1988; van Noort et al., 1988; Waite, 1988). Fulvic acids also exhibit considerable reducing ability towards metal ions (Skogerboe & Wilson, 1981; Waite & Morel, 1984).

Humic substances are readily adsorbed on the surfaces of iron oxides and aluminosilicates, and thus have a significant impact on the subsequent adsorption of inorganic cations and anions (Greenland, 1971; Orlov et al., 1973; Davis & Leckie, 1978; Tipping, 1981; Davis, 1982; Davis, 1984). The more aliphatic humic fractions may penetrate

the interlamellar layers of clays (Inoue et al., 1990). Further, humic substances are involved in the development of soil structure, especially Podzols (although the exact nature of their involvement is the subject of debate) (Anderson et al., 1982; Farmer et al., 1983).

1.3 EXTRACTION OF HUMIC SUBSTANCES

Ideally, humic substances should be studied in an unaltered state. Unfortunately, any extraction procedure is likely to cause some (unquantifiable) change in the organic material. *In situ* measurements are preferable, but few techniques provide adequate sensitivity. In general, extraction of humic substances from soil involves leaching with alkali to dissolve humic and fulvic acids, followed by acidification to precipitate the humic acid. The advantages and disadvantages of the various techniques employed are discussed in Chapter 5.

A problem which must be addressed is that different operationally defined fractions of 'humic acid' and 'fulvic acid' are isolated by each method, in addition to different amounts of associated 'impurities' (carbohydrates, lipids, and proteinaceous compounds). Various treatments have been applied to "purify" humic substances, *viz:* boiling in water to remove polysaccharides, polypeptides, and small amounts of phenolic acids and aldehydes (Haworth, 1971); hydrolysis with strong acid (6 mol L^{-1} HCl) to remove lignin, sugar, and protein moieties and metals (Barker et al, 1965; Atherton et al., 1967; Riffaldi & Schnitzer, 1973; Schnitzer & Preston, 1983; Preston & Schnitzer, 1984; Saiz-Jimenez & De Leeuw, 1987a); extraction with phenolic solvents to remove proteinaceous materials which are hydrogen-bonded to humic molecules (Simonart et al., 1967; Biederbeck & Paul, 1973); and dialysis to remove metal impurities, with a concomitant loss of low molecular weight species. However, purification may well result in alteration of the 'humic' material. Further, since there is general agreement that humic substances are mixtures of compounds, there is no reason why the term 'humic substance' should be applied only to the material which is left behind after the extraction of one or more 'impurities' and not to the 'unpurified' material (Wershaw, 1986).

1.4 PROPERTIES OF HUMIC SUBSTANCES FROM DIFFERENT ENVIRONMENTS

Humic substances from different environments (soil-derived, aquatic, marine, sedimentary) may have different structural characteristics. Numerous factors are involved in determining the composition of a particular humic sample, including the predominant flora, the effects of microbial activity, and geographical factors such as climate.

Malcolm (1990) identified definitive compositional differences for both humic and fulvic acids from soil, stream, and marine environments. There were also significant differences between humic and fulvic acids from each particular source. According to Malcolm (1990), the major compositional features which contributed to the observed differences were the amounts and composition of saccharide, phenolic, methoxyl, aromatic, hydrocarbon, amino acid, and nitrogen moieties. There could also be considerable variation in the composition of humic substances from the same environment. For example, humic substances from Podzol B_h horizons are reported to be relatively aromatic due to the selective precipitation of these moieties as they percolate through the soil profile (Patience & Wilson, 1990).

The different methods used to extract humic substances from soils and aquatic sources could account, in part, for the compositional differences observed (Lobartini et al., 1989; Chapter 5). However, some similarities in the features of these samples may be expected because much of the humic substances in freshwaters are derived from terrestrial sources (Wilson et al., 1983b).

Marine humic substances are likely to be significantly different from those derived from soils or freshwaters because lignin is not available as a precursor in seawater (Stuermer & Payne, 1976; Hatcher et al., 1981b). Marine humic acids are predominantly aliphatic and are thought to be derived from algal and/or bacterial residues (Nissenbaum & Kaplan, 1972; Hatcher et al., 1980b; Sohn & Weese, 1986). For sedimentary humic substances, Orem and Hatcher (1987) identified two types of materials: one type was comprised of carbohydrate and paraffinic structures, while the other was dominated by aromatic and paraffinic moieties. These authors proposed that the dominance of one of these

structural types was dependent on the oxidizing/reducing nature of the sediment and on the source of the detrital organic matter. Ishiwatari (1973) found that humic acids from Recent and lacustrine sediments had a cyclic structure (40 - 50% of the total carbon) which was alicyclic rather than aromatic.

1.4.1 Standard Humic Samples (IHSS)

A range of "standard" and "reference" humic substances (both soil-derived and from aquatic sources) are available from the International Humic Substances Society (IHSS). Details of the source and extraction method of these samples are readily available. Although extraction of these samples may have caused some alteration of the samples (and they may not be representative of humic substances from a particular environment) they at least allow results obtained by different workers to be compared.

1.5 ELEMENTAL AND FUNCTIONAL GROUP COMPOSITION OF HUMIC SUBSTANCES

The composition of fulvic acids (weight percent) is typically: C, 40 - 50%; O, 44 - 50%; that for humic acids is: C, 50 - 60% and O, 30 - 35%. Both humic substances contain H, 4 - 6%; N, 2 - 6% and S, 0 - 2% (Stevenson, 1982). (Huffman & Stuber (1985) have reviewed techniques used for the elemental analysis of humic substances.) For aquatic fulvic acids, Abbt-Braun et al. (1989) reported ratios of: C/H = 1; O/C = 0.6 - 0.7; and, N/C = 0.01 - 0.02.

Functional group analysis to determine the oxygen-containing functional groups is subject to cumulative errors. For example, phenol content is usually calculated as total acidity minus carboxyl content; alcoholic hydroxyl content is then calculated as total hydroxyl groups minus the phenolic content. For aquatic fulvic acids Abbt-Braun et al. (1989) determined approximately one carboxyl group per 7 to 9 carbon atoms, and one phenolic group per 20 to 30 carbon atoms. Suwannee River fulvic acid (IHSS) was reported to contain less than five carboxyl groups per molecule (Aiken & Malcolm, 1987).

Using NMR and IR spectroscopy, Leenheer et al. (1987) found that aromatic ketone groups comprised the majority of the carbonyl content of IHSS standard aquatic humic and fulvic acids. There was approximately one ketone group per monocyclic aromatic ring in all samples. It was proposed that such aromatic ketone groups could be the point of attachment between aliphatic and aromatic moieties in aquatic humic substances (Leenheer et al., 1987). ^{13}C NMR of methylated humic substances has enabled hydroxyl functionalities to be characterized (Mikita et al., 1981; Wershaw et al., 1981; Thorn et al., 1987b). This technique allowed resolution between methyl esters of carboxylic acids, of phenolic hydroxyl groups, and of phenolic hydroxyl groups adjacent to two substituents.

Acidic functional groups in humic substances are predominantly of three types: carboxyl groups on aromatic rings *ortho* to phenolic groups, more weakly acidic carboxyl groups, and phenolic hydroxyl groups (Borggaard, 1974). According to Perdue (1978), at least one-third of phenolic hydroxyl groups are not *ortho* to carboxyl groups.

Schnitzer (1985) has reviewed the nature of nitrogen in humic substances. Only 35 - 50% has been characterized. The characterized nitrogen is comprised of amino acids, amino sugars, ammonia, and heterocyclic moieties. The predominant amino acid moieties identified in hydrolyzed humic substances and natural waters are glycine, aspartic acid, alanine, serine, and glutamic acid (Tuschall & Brezonik, 1980; Lytle & Perdue, 1981). Histidine may be present in significant amounts in soil humic acids (Malcolm, 1990).

Although nitrogen-containing moieties are minor components of humic substances, they may have a significant influence on their metal binding properties (Buffle et al., 1990b; Chapter 6). Analysis of Suwannee River humic substances indicated that fulvic acid would contain one amino acid residue per 10 molecules, while humic acid would contain at least one amino acid residue per molecule (Thurman & Malcolm, 1989).

Any compositional differences between humic and fulvic acids may be determined to some extent by the method used to isolate or separate these fractions. For example, carbohydrates are soluble in acid and hence are included in the fulvic acid fraction; whereas nitrogenous and polyphenolic compounds tend to precipitate with the humic acids (which generally have a higher C/N ratio) (Hatcher et al., 1983; Gadel & Bruchet, 1987).

Differences in the composition of amino acid residues in humic and fulvic acids from the Suwannee River were ascribed to the separation of these humic fractions by precipitation at pH 1.0 (Thurman & Malcolm, 1989).

1.5.1 Chemical Degradation Studies

Stevenson (1982) has reviewed studies on determination of the composition of humic substances. This earlier work focussed on the use of chemical degradation techniques such as: oxidation (e.g. alkaline $KMnO_4$, peracetic acid, CuO-NaOH, alkaline nitrobenzene, nitric acid, H_2O_2) (Schnitzer & De Serra, 1973; Slawinska & Slawinski, 1975a,b); reduction (e.g. zinc dust distillation, Na-amalgam); hydrolysis (e.g. acid, base, water) (Swift & Posner, 1972; Riffaldi & Schnitzer, 1973); depolymerization (Almendros et al., 1987); microbial degradation (Mathur & Paul, 1966, 1967a,b; Ladd & Brisbane, 1967; Mathur, 1971; Bertino et al., 1987); pyrolysis (MacCarthy et al., 1985; Schulten et al., 1987); low temperature ashing (De Kimpe & Schnitzer, 1990); and functional group derivatization (Leenheer & Noyes, in press).

A problem with each of these techniques is that some of the isolated degradation products may have been generated by the decomposition process itself. With milder procedures the yield of identifiable products is very low, while the more drastic methods may cause such extensive decomposition (to acetic acid, CO_2, and H_2O) that any useful relationship to the original humic structure is lost.

1.5.2 Spectroscopic Techniques

Nuclear Magnetic Resonance Spectroscopy (NMR)

More recently, nondestructive NMR techniques have been applied extensively to compositional analysis of humic substances. There has not been good agreement between interpretations based on classical degradation studies and ^{13}C NMR. Hatcher et al. (1981a) concluded that chemical degradation techniques provide no information on the nature of aliphatic structures in humic acids; hence the importance of these components was

underestimated prior to NMR analysis. NMR generally gives a lower estimate of the aromaticity of humic substances than do the degradative techniques. Determination of carbon aromaticity has been the focus of many studies because it is thought to be the most sensitive source determinant for humic substances (Hatcher et al., 1980a). At first sight, NMR appears to be an ideal technique for studying humic substances; both solid and solution samples can be used, and no manipulation of the sample is required (Barron et al., 1980). However, as expected for humic substances, interpretation of the data is not a simple matter.

The NMR spectra of humic substances are poorly resolved and generally show only broad bands. They do, however, allow resolution of at least four types of components, *viz:* aliphatic carbon, aromatic carbon, carbon attached to oxygen, and carbon in carboxyl groups. Peak broadening arises from the extreme molecular heterogeneity of the samples, and the presence of paramagnetic ions and free radicals (Dereppe et al., 1980). The relative signal intensity of each fraction may not be quantitatively represented because of inhomogeneous relaxation times for the heterogeneous humic molecules (the slower relaxing nuclei make a smaller contribution to the total spectrum than do the faster relaxing ones). The relaxation times for aromatic carbons are especially long (Wilson, 1981). Several methods have been applied to overcome this problem such as choice of contact time or recycle delay, or by curve fitting relaxation behaviour to obtain the signal intensity in the absence of relaxation effects (Wilson et al., 1983a, 1987; Preston & Blackwell, 1985). Spin-lattice relaxation times are reduced by paramagnetic ions (Wilson et al., 1983a; Pfeffer et al., 1984). Treatment with sodium dithionite may minimize this interference (Vassallo et al., 1987).

Some complex pulse sequences have been used, including 2-D NMR techniques (Buddrus et al., 1989). Although these approaches may produce the correct representation of *observable* carbons, they cannot indicate how much of the humic carbon is actually being detected. A further complicating factor is that phenolic carbon signals may be shifted downfield so that they overlap with those for carboxyl groups, thus resulting in an overestimation of carboxyl groups and an underestimation of phenolic hydroxyl groups (Schnitzer & Preston, 1986).

"Quantitative" ^{13}C NMR measurements have been used to calculate analytical constraints on the structural features of humic substances (Perdue, 1984). For soil humic substances, Wilson et al. (1987) calculated that the *maximum* percent aromatic carbon was 63% for humic acid, and 30% for fulvic acid. It is likely that the relative importance of aliphatic and aromatic moieties in humic substances will continue to be the subject of debate (Ruggiero et al., 1979; Wilson & Goh, 1981). NMR studies have provided evidence for substantial amounts of branched chain structures in the aliphatic moieties of humic and fulvic acids (Hatcher et al., 1980b; Thorn, 1987).

Pyrolysis - Mass Spectrometry

Pyrolysis of carbohydrates yields anhydrosugars, furans, and carbonyl compounds; proteins produce pyrroles, indoles, and nitriles; polyhydroxyaromatics generate phenolic compounds, and methoxyphenols are characteristic products from lignins (Gadel & Bruchet, 1987). Schulten et al. (1987) observed furan, phenol, methoxyphenol, and dimethoxyphenol moieties in the pyrolysis field ionization mass spectra for aquatic humic and fulvic acids. MacCarthy et al. (1985) reported significant amounts of carbohydrate and phenolic components in the pyrolysis products of fulvic acids, while saturated and unsaturated hydrocarbons were predominant for humic acids. This technique has also indicated that aliphatic polycarboxylic acids may be important components of soil humic substances (Bracewell et al., 1980; Wilson et al., 1983b).

Other Spectroscopic Techniques

Other nondestructive techniques such as UV-visible, infrared and fluorescence spectroscopy have been applied to humic substances. However, the spectra are generally featureless and, other than illustrating the extreme heterogeneity of these materials, they provide little structural information (MacCarthy & Rice, 1985). The ratio of absorbances at 465 nm and 665 nm (the "E_4/E_6 ratio") has been used as a measure of the degree of 'condensation' (conjugation) of aromatic humic constituents (Ghosh & Schnitzer, 1979); a low value indicates a high degree of 'condensation'. Its interpretation has, however, been

questioned (Chen et al., 1977). Recently, it has been suggested that fluoresence emission spectra may be useful in resolving condensed aromatic structures (Miano et al., 1988).

The 'true' proportion of aromatic moieties will be dependent on the source of the humic substance (soil, aquatic, marine, or sedimentary). For example, humic acids from volcanic ash soils were reported to be highly aromatic (Hatcher et al., 1989); whereas for humic acids from other soil types, aromatic moieties may only be a minor component (Wilson & Goh, 1977). The particular extraction method employed may also have some effect. For example, soil humic substances are usually extracted with strong alkali; this could generate aromatic species (Tinsley & Salam, 1961; Reuter et al., 1983). In contrast, aquatic humic substances (which are generally reported to be less aromatic (Malcolm, 1985)) are isolated by preconcentration on XAD resins. Aromatic moieties are strongly adsorbed on XAD resins and hence may not be totally eluted with the 'humic' fraction (Chapter 5).

Further investigations on the structural characterization of humic substances are required. The use of IHSS humic samples could help to distinguish between 'real' and 'technique dependent' differences in the determined composition. The development of new techniques in recent years has challenged traditional concepts about humic substances. For example, by use of pyrolysis and ^{13}C NMR Nip et al. (1986, 1987) identified insoluble, nonhydrolyzable, highly aliphatic biopolymers in algal cell walls and terrestrial plants. These could be major precursors for aliphatic moieties in humic substances (Tegelaar et al., 1989). Consistent with this hypothesis, pyrolysis of the resistant humic acid fraction has yielded mainly homologous series of aliphatic hydrocarbons (Saiz-Jimenez & De Leeuw, 1987a,b). Algal and microbial sources could also make a significant contribution to aliphatic moieties in terrestrial humic substances (Hatcher et al., 1981b).

Lignin has traditionally been regarded as a major source of the aromatic moieties in soil humic substances (Stevenson, 1982). However, by use of NMR techniques Wilson et al. (1986) identified aromatic components in soils without a lignin input, indicating that it need not be a necessary precursor.

1.6 STRUCTURAL MODELS FOR HUMIC SUBSTANCES

Many attempts have been made to develop structural models for humic substances. Any model must be consistent with the known properties of humic substances, such as: resistance to microbial degradation; solubility; aggregation/dispersion phenomena; metal chelation, base exchange, affinity for proteins, free radical content, and association with hydrophobic moieties (Leenheer et al., 1989b). Several workers have published detailed specific molecular structures (e.g. Buffle, 1984; Leenheer et al., 1989b). However, given the heterogeneity of humic substances, the relevance of an 'average' or 'representative' structure must be questioned. An understanding of the likely macromolecular structure resulting from associations of individual molecules may be more pertinent.

1.6.1 Polymeric Models

Early polymeric models considered humic acids to be highly cross-linked, predominantly aromatic polymers with covalent bonds (such as carbon-carbon, ether and ester linkages) joining the monomeric units together into large, high molecular weight macromolecules (Dubach & Mehta, 1963; Ogner & Schnitzer, 1971). Cheshire et al. (1967) proposed that humic acid possesses a chemically resistant polycyclic aromatic core to which polysaccharides, proteins, and phenolic acids could be linked; these peripheral units may also be linked to each other. Minderman (1979a,b,c) considered humic acids as heterogeneous polymers of mixed esters or tannins.

Much of this earlier work assumed that phenolic and benzene carboxylic acids were the major 'building blocks' of humic substances, held together by hydrogen bonding (Schnitzer & de Serra, 1975; Schnitzer, 1977). Chemical degradation techniques had produced results consistent with this concept (Neyroud & Schnitzer, 1975).

1.6.2 Random Coil Models

These models attempt to explain the changes in macromolecular configurations of humic molecules in solution. Such changes are dependent on the sample concentration, pH and ionic strength. It is proposed that humic and fulvic acids behave as "rigid spherocolloids" at high sample concentrations, low pH, or high ionic strength, but are "flexible linear colloids" at low sample concentrations provided the pH is not too low or the ionic strength is not too high (Cameron et al., 1972b; Chen & Schnitzer, 1976b; Ghosh & Schnitzer, 1980).

1.6.3 Aggregation Model

A hierarchical model for humic acid has been proposed by Wershaw et al. (1977). It was suggested that simple phenolic, quinoid, and benzene carboxylic acid groups represent the lowest level of structural elements. These groups are covalently bound into small molecules; molecules of similar chemical structure are then linked together by weak covalent and noncovalent bonds to form "homogeneous" aggregates. Two or more different types of aggregates may link together to form mixed aggregates. The bonding mechanisms proposed were: hydrogen bonding; charge transfer complexation between free radicals; π-bonding between planar aromatic ring structures; and metal ion bridging between humic molecules. The mechanisms involved in the aggregation/disaggregation of such structures would be determined by the distribution of charge and functional groups on the exposed surfaces of the aggregates (Wershaw & Pinckney, 1973b).

1.6.4 Micelle Model

Micellar aggregates are the most recent concept for the structure of humic acids (Wershaw, 1986). The main evidence for such structures comes from the ability of humic substances to enhance the aqueous solubility of nonpolar substrates (Section 1.10.2), the surface activity of humic molecules (Tschapek et al., 1981; Hayase & Tsubota, 1983), and their catalytic properties (Perdue & Wolf, 1982; Perdue, 1983).

Surface activity in general is characteristic of amphiphilic molecules of intermediate (several hundred Dalton) molecular weight. The critical micelle concentration (cmc) can be

detected in surfactant solutions by observation of discontinuities in concentration-dependent properties such as surface tension, conductance, and osmotic pressure. However, due to the heterogeneous nature of humic substances, these properties do not change abruptly with change in concentration (Section 1.10.2).

Wershaw (1986) proposed that humic acids were comprised of aggregates which resemble micelles, having a hydrophilic exterior (with which metal ions can interact) and a hydrophobic interior (into which hydrophobic compounds can partition). Hydrophobic regions could consist of polar groups bound together by hydrogen bonding such that the resulting aggregate is reasonably hydrophobic. More recently, Caceci and Billon (1990) have obtained evidence (from photon correlation spectroscopy) for large aggregated structures (0.05 to 0.20 μm in diameter) in humic acid solutions. They concluded that although such structures could be responsible for the 'micelle-like' properties of humic substances, they were better described as strongly bound (perhaps covalently) stable agglomerates rather than transient aggregates (such as micelles and vesicles) which would be in dynamic equilibrium with monomeric species.

Evidence for Hydrogen Bonding in Humic Substances

It is generally accepted that humic substances are extensively aggregated in aqueous solution (Biederbeck & Paul, 1973; Brown & Leenheer, 1989). The high proportion of acidic functional groups in humic substances indicates that hydrogen bonding will be an important mechanism in both inter- and intramolecular aggregation (Wershaw & Pinckney, 1977, 1980). The extent of hydrogen bonding will be a function of pH, the oxidation state of the humic molecules and the solvation properties of the solvent. Using UV-visible absorption spectroscopy, Ghosh and Schnitzer (1979) concluded that there was extensive hydrogen bonding in aqueous fulvic acid solutions at pH 2.0.

Evidence for hydrogen bonding is provided by carboxyl methylation of humic substances; this eliminates hydrogen bonding resulting in a reduction in molecular size (Bartle et al., 1987) and release of significant amounts of "impurities" (Schnitzer & Ogner,

1970; Schnitzer & Neyroud, 1975). The reduction in aggregation on methylation of humic substances also improves the resolution of ^{13}C NMR spectra (Gonzalez-Vila et al., 1983).

Humic molecule aggregates are smaller in aprotic solvents, such as dimethyl sulphoxide and N,N-dimethyl formamide, (Wershaw & Pinckney, 1977); solvation of the acidic groups inhibits hydrogen bonding.

However, other authors have questioned the importance of hydrogen bonding and have proposed that other mechanisms could explain the high molecular weights measured for humic substances. For example, Hatcher et al. (1989) suggested that the macromolecular structure of humic substances could arise from biphenyl-linked benzene carboxylic acids.

1.7 MOLECULAR WEIGHT DETERMINATION

Techniques for determination of the molecular weight or size of humic substances are discussed in Chapter 5. Each method is operationally defined and results in a different estimate of this parameter. A cause of these discrepancies could be the conditions under which the measurements are made. As noted above, both the degree of aggregation of humic substances and the configuration of individual molecules will vary with pH, ionic strength, and the concentration of the sample. Some techniques may give estimates of molecular sizes or weights which are representative of aggregated structures, e.g. gel permeation chromatography (Orlov et al., 1975) and ultrafiltration (Buffle et al., 1978b; Aiken, 1984); in contrast, others may respond to individual humic molecules, e.g. flow field-flow fractionation (Beckett et al., 1987), and vapour pressure osmometry (Aiken & Malcolm, 1987).

1.8 COMPLEXATION OF PROTONS AND METAL IONS BY HUMIC SUBSTANCES

A knowledge of the acid-base properties of humic substances is fundamental to an understanding of their interactions with metal ions, and their solubility characteristics. An understanding of these properties is of considerable environmental importance; the metal buffering action of humic substances is essential for maintenance of life in natural systems (Buffle et al., 1990a,b).

A description of the protonation equilibria of humic substances requires information on the concentration of acidic functional groups and their respective pK_a values. Neither of these parameters are known for humic substances. Effects arising from the polyfunctionality and polyelectrolytic character of humic substances are difficult to resolve. Therefore, models which have been developed to describe both protonation and metal ion complexation equilibria of humic substances are usually formulated in terms of either "heterogeneity" or "charge" effects. Due to the extreme heterogeneity of humic substances, their titration curves are smooth and featureless with only one equivalence point being observed at $pH \geq 8$ Hence, almost any mathematical model, with several adjustable parameters, can be used to empirically fit the data (Cabaniss et al., 1984). Indeed, metal complexation by humic substances is described equally well by either 'discrete' or 'continuous multiligand' models (Dzombak et al., 1986; Giesy et al., 1986). Therefore, "goodness-of-fit" is not a valid criterion to judge whether a particular model is chemically reasonable. Models to describe the protonation and metal complexation equilibria of humic substances have been extensively reviewed (Perdue & Lytle, 1983b; Buffle, 1984; Cabaniss et al., 1984; Fitch & Stevenson, 1984; Ruzíc, 1984; Perdue, 1985; Sposito, 1981, 1986; Dzombak et al., 1986; Fish et al., 1986; Turner et al., 1986; Gamble & Langford, 1988). Only a brief description of the various models is now presented.

1.8.1 Discrete Binding Site Models

On the basis of pH titration data alone, it is not possible to distinguish a polyprotic acid from a mixture of monoprotic acids (Perdue, 1984); hence, acidic functional groups of humic substances can be treated as a mixture of monoprotic acids (Sposito & Holtzclaw, 1977; Sposito et al., 1977). Interpretation of humic substance titration curves in terms of this model can be misleading. For example, if the humic substance data can be described by a mixture of, say, 4 monoprotic acids, some authors have been tempted to conclude that humic substances must contain 4 'classes' of binding sites (Wilson & Kinney, 1977; Choppin & Kullberg, 1978). This deduction is not valid. The pK_a values determined for the humic substance titration curve are curve fitting parameters with no chemical significance (Perdue, 1985).

Scatchard analysis of humic substance titration data has indicated the presence of two 'classes' of discrete binding sites (Stevenson, 1982). In this approach binding is described by a formation function: $\upsilon = \frac{\text{concentration of bound metal ion}}{\text{total number of binding sites}}$. When all binding sites are identical, υ is related to the overall binding constant, K_o, by: $\upsilon = K_o[M]/(1+K_o[M])$. Thus, the slope of a Scatchard plot (υ/[M] *versus* υ) yields K_o. However, the division of Scatchard plots into two straight line segments is arbitrary; 'additional sites' can be found by selecting additional linear sections of the plot (Fitch & Stevenson, 1984). Further, in simple systems such as Cu(II)-polyaspartic acid, Scatchard analysis has indicated the presence of several 'classes' of binding sites even though only a single site is most likely to be involved (Tuschall & Brezonik, 1983b).

Discrete models should not be used even to compare the properties of different samples under identical experimental conditions (Buffle, 1988). However, Fish et al. (1986) considered this approach to be the most useful way to model metal complexation by humic substances because of the ease with which discrete ligands can be incorporated into computer programs for speciation calculations.

1.8.2 Intrinsic Binding Site Models

In these models all acidic functional groups are considered to be in identical electrostatic environments in the fully protonated molecule (Dempsey & O'Melia, 1983; Varney et al., 1983). That is, the "intrinsic stability constant", pK_{int}, corresponds to a state of zero charge on a molecule containing chemically identical acidic groups. Dipolar groups are ignored. Differences in pK_a values are ascribed to increasing electrostatic effects from adjacent ionized substituents as deprotonation progresses. The apparent protonation constant is assumed to increase with increasing degree of dissociation, caused by the accumulation of charge on the molecules.

An equation frequently used to describe humic substance titration data is a modified Henderson-Hasselbalch equation: $pH = pK_{int} + n[\log (\alpha/(1 - \alpha))]$; where n represents the degree to which the pK_a values are modified by electrostatic effects, and α is the degree of ionization of humic acidic functional groups.

These models are based on the theory for simple polyelectrolytes in which protonation can be described by apparent thermodynamic functions for an 'average group' on the polyelectrolyte. However, 'average constants' are not pertinent to multiligand mixtures of nonidentical ligands, hence the 'stability constants' and 'binding site concentrations' obtained by these methods are only curve fitting parameters. Recently, van den Hoop et al. (1990) have established (*via* conductimetric titrations) that theoretical results for linear polyelectrolytes, such as polymethacrylate, are not strictly applicable to humic acids. That is, humic acids should *not* be considered as linear polyions with randomly distributed charges. It has been suggested that the term 'oligoelectrolytes' may be the best description of humic substances (Aiken & Malcolm, 1987).

By correcting for Donnan potential terms, this model has been extended to include a "gel phase" (Marinsky et al., 1982b; Marinsky & Ephraim, 1986; Ephraim et al., 1986). However, the validity of such "corrections" has been questioned (Cabaniss et al., 1989) and the presence of chemically identical groups was still assumed. By application of Debye-Hückle theory, Tipping et al. (1990) have attempted to separate electrostatic effects from those arising from the heterogeneity of ionizable groups.

1.8.3 Continuous Distribution Models

These models are based on approximate analytical expressions for solution of the integral equation defining the titration curve, such that a continuous distribution of binding sites is generated. The majority of models assume that the individual binding site concentrations are normally distributed with respect to a finite number of mean pK_a values (Gamble, 1970, 1972; Burch et al., 1978; Perdue & Lytle, 1983a,b; Perdue et al., 1984; Dobbs et al., 1989a,b). For a simple normal distribution of binding sites:

$$\frac{C_i}{C_L} = \frac{1}{\sigma\sqrt{2\pi}} \exp\left[-\frac{1}{2}\left[\frac{\mu - pK_i}{\sigma}\right]^2\right] dpK;$$ where $\frac{C_i}{C_L}$ is the mole fraction of binding sites in the interval dpK whose proton dissociation constant is expressed as a negative logarithm (pK_i), and σ is the standard deviation for the distribution of pK_i values about the mean pK value (μ) for the mixture of binding sites.

The assumption of a normal (Gaussian) distribution, however, may not be valid (Fish et al., 1986). Leuenberger and Schindler (1986) proposed the use of "integral pK spectrometry" in which a continuous pK spectrum of "selectable resolution" was obtained.

Although these models take the important step of acknowledging the heterogeneity of humic substances, characterization of humic functional groups suggests that such heterogeneity is probably not as great as that assumed by these models. Further, although these models can provide a reasonable fit to the humic substance titration data, their predictive capabilities are not good. For example, they cannot predict the effects of sample concentration, ionic strength, temperature, or metal binding on the protonation equilibria.

Continuous distribution models have also been used to describe metal ion complexation by humic substances (Perdue & Lytle, 1983a; Susetyo et al., 1990). A limitation of these models is that they do not provide data which is compatible with chemical speciation computer programs. Further, the majority of workers have ignored the competition between protons and metals for the same binding sites (e.g. Perdue & Lytle, 1983a). Recently, however, Dobbs et al. (1990) have attempted to account for this. Of course in an environmental situation, in addition to protons, several metal ions will simultaneously

compete for the humic binding sites; the presence of other competing ligands must also be considered.

Another important aspect is the available "analytical window" (Altmann & Buffle, 1988; Buffle, 1988). The number and concentration of binding sites determined by these models, and their stability constants, will be dependent on the portion of the complete titration curve (the analytical window) for which data are available.

1.8.4 Site Occupation Distribution Functions

The most rigorous descriptions to date of protonation and metal ion complexation equilibria for humic substances have been formulated by Buffle and coworkers. These models aim to account for the following properties of humic substances: (i) polyfunctionality (the presence of different coordinating sites on the same molecule), (ii) polyelectrolytic character (the charge density on each molecule due to ionized functional groups), and (iii) conformational factors (reactions on surfaces or within gels, and the formation of aggregates).

The titration curves for humic substances cannot be resolved adequately to allow assignment of discrete pK values. Buffle and coworkers have described protonation and metal ion complexation equilibria in terms of distribution functions ('site affinity', 'differential equilibrium', and 'site occupation' distribution functions) (Buffle & Altmann, 1987; Altmann & Buffle, 1988). An important aspect of this approach is transformation of the experimental data into a *normalized* format. This transformation is purely mathematical and is not dependent upon an *a priori* hypothesis as to the physicochemical reasons for the shape of the resulting titration curve (Buffle et al., 1990a). This allows comparison of the complexation properties for different samples, and for one sample measured under different conditions (e.g. pH, ionic strength). Further, complexation behaviour can be predicted outside the measured experimental conditions.

The dependent variable of the site occupation distribution function (SODF), ω^*, is

defined as: $\omega^* = \frac{-d[M]_b/\{S\}_t}{d\log K^*}$; where $[M]_b$ is the concentration of bound metal ion, $\{S\}_t$ is the concentration of binding sites, and K^* is the weighted arithmetic mean of the conditional equilibrium constants for all sites. The utility of the SODF derives from the analogy between ω^* and the overall metal buffer intensity of the system, β, defined as: $\beta = \frac{d(C_m/\{S\}_t)}{d\log[M]}$; where C_m is the total metal ion concentration, and [M] is the free metal ion concentration. Under conditions where $[M] \ll C_m$ (hence $C_m \approx [M]_b$), there is a parallel between the functions $\beta = f(\log [M])$, and $\omega^* = f(\log K^*)$.

Using the SODF, Buffle et al. (1990a) separated humic binding sites into two classes: (i) "major" sites, which are present in large proportions, e.g. carboxylate and phenolate groups, and (ii) "minor" sites, which are present in only small amounts (not more than *ca.* 10% of the total sites), e.g. nitrogen and sulphur containing moieties. The minor sites were further subdivided into "dominant" and "background" sites. The dominant sites are chemically homogeneous and are present at higher concentrations than the underlying continuum of background sites. The continuum of background sites was proposed to be responsible for maintaining the metal buffering action of humic substances over many orders of magnitude (Buffle et al., 1990b).

A major advantage of this approach is that it yields parameters which can be used in chemical speciation programs.

However, the need for such a detailed analysis has been questioned. Some authors have taken the opposite approach, ignoring both electrostatic effects and the heterogeneity of humic substances (i.e. they considered a set of distinct constants rather than a distribution of constants) (Paxeus & Wedborg, 1985; Gregor & Powell, 1988b). Given our limited knowledge of the structure of humic substances, these authors considered a description of their acid-base properties in terms of a limited number of "chemically reasonable" parameters to be a more realistic approach. Even those workers that have rigorously analyzed humic protonation equilibria conclude that an equivalent analysis of metal binding is not feasible (De Wit et al., 1990; Driscoll & Schecher, 1990).

For a complete understanding of the complexation properties of humic substances, information is required on (i) the thermodynamic stability of the complexes, (ii) the binding capacity as a function of pH and ionic strength, (iii) the lability of the complexes, and (iv) the kinetics of complex formation and dissociation. None of these parameters has been adequately measured (Stevenson & Vance, 1989).

1.8.5 Nature of Metal Binding Sites in Humic Substances

Acidic oxygen-containing functional groups, such as carboxyl and phenolic moieties, are the sites in humic substances most likely to be involved in the complexation of metal ions. Benzene carboxylic acids have been recovered in significant amounts from humic substances by various chemical degradation techniques (Stevenson, 1982). On this basis salicylate and phthalate moieties have been considered as the predominant oxygen-containing functional groups in humic substances. Erroneously, some authors have assumed that the numerically dominant sites will be the most important for metal ion complexation (Murray & Linder, 1983; Goncalves & Mota, 1987).

The apparent conditional stability constants measured for metal complexation by humic substances have also been used as indicators of the types of binding sites present. As noted above, this assumption has no chemical validity.

By comparison of Cu(II) binding curves for fulvic acid with those for discrete ligands, Cressey et al. (1983) proposed an aliphatic carboxylate mode of coordination for fulvic acids in weakly acidic solution. These authors demonstrated that salicylate and phthalate moieties bind Cu(II) far too weakly to be considered as significant humic complexation sites in near-neutral solution at high dilution.

Other minor structural components such as amino groups, S-donors, and 1,2-dihydroxy moieties may contribute to complexation of metal ions by humic substances. Electron spin resonance spectroscopy has revealed the involvement of both oxygen and nitrogen atoms in Cu(II) coordination (Senesi et al., 1985, 1989).

1.9 EVIDENCE FOR SORPTION OF HYDROPHOBIC COMPOUNDS BY HUMIC SUBSTANCES

As hydrophobic pollutants have the greatest potential for bioaccumulation and transfer to humans *via* food chains, the behaviour of these chemicals in soils and natural waters is of considerable environmental interest. Nonpolar pesticides are extremely stable compounds with a residual life of 5 to 30 years; DDT is one of the most persistent (Pierce et al., 1971). It has been estimated that biota contain less than $1/30^{th}$ of one year's production of DDT in the mid-1960s (Woodwell et al., 1971). Therefore, most of the DDT manufactured must have either been degraded to innocuous compounds or sequestered in places where it is not freely available to biota. Woodwell et al. (1971) favour the latter assumption. However, it is unclear how, or where, DDT residues are held. Perhaps humic substances play an important role in this process. For example, the concentration of DDT in water is increased 16 000 times in the presence of humic colloids (Poirrier et al., 1972). Binding of xenobiotics to humic substances could be utilized as a means for decontamination.

Association of hydrophobic organic compounds with humic substances has been described in terms of a distribution coefficient (K_d). K_d is defined as: $\frac{X/M}{C_{eq}}$. Where X is the mass of compound sorbed, M is the mass of sorbent, and C_{eq} is the equilibrium concentration of sorbate in the aqueous phase. This value can also be expressed on an organic carbon basis (K_{oc}) or on an organic matter basis (K_{om}): i.e. $K_{oc} = K_d/f_{oc}$, $K_{om} = K_d/f_{om}$, where f_{oc} and f_{om} are the weight fraction of sorbent comprised of organic carbon or organic matter respectively (Garbarini & Lion, 1986).

In general, the extent of association of a compound with humic matter is directly related to the value of its octanol-water partition coefficient (K_{ow}) and inversely proportional to its water solubility. K_{ow} is the equilibrium concentration of the compound in octanol divided by the concentration in water. It has been reported that the solubility enhancement of various nonionic solutes by dissolved organic matter is significant only for solutes which have a water solubility at least two orders of magnitude lower than the concentration of dissolved organic matter (Kile & Chiou, 1989a). However, the environmental situation is

extremely complex and involves many interrelated and interdependent factors, *viz:* the particular fraction of humus studied; the composition of the humic fraction (molecular size, polarity, configuration of molecules); the source of humic matter (aquatic or soil-derived) and the extraction technique employed; pH; ionic strength; and, the presence of cations. The effect of these individual parameters is now discussed.

1.9.1 Effect of Source and Fraction of Humic Material

Humic acid has a greater capacity to sorb hydrophobic compounds than does fulvic acid. Similarly, soil-derived humic substances have a greater capacity than do those from aquatic sources (Kile & Chiou, 1989a). Mingelgrin and Gerstl (1983) found literature values of K_{om} for many nonionic compounds that varied by an order of magnitude between soils. This implies that the relationship between K_{om} and a solute's K_{ow} value is a function of the nature of the organic carbon involved. It also limits the precision with which the expected uptake of these nonpolar compounds by soil organic matter can be estimated.

Ballard (1971) studied the role of humus in controlling the movement of DDT through a forest soil. In DDT treated extracts 91% of added DDT was recovered in the humic acid fraction, and 9% in fulvic acids and water. Further evidence for the specificity of association of lipid-soluble substrates with particular humic fractions comes from a study by Chiou et al. (1986). In this, the increase in water solubility of some organic pollutants in the presence of humic and fulvic acids from soils and aquatic sources was compared with that in the presence of synthetic organic polymers (high molecular weight poly(acrylic)acids). The constants for hydrophobic substrate association with soil humic acid were approximately 4 times greater than with soil fulvic acid, and 5 to 7 times greater than with aquatic humic and fulvic acids. No significant solubility enhancement was observed with the synthetic polymers.

Carter and Suffet (1982) observed a range of K_{oc} values for DDT sorption by dissolved humic and fulvic acids from different sources. The largest differences occurred between humic and fulvic acids. It was not possible to correlate the extent of DDT association with measurable characteristics of the dissolved humic materials. Structural differences between similar macromolecules may alter partition coefficients by a factor of 10

or more (Chiou et al., 1986). The water solubility enhancement of nonionic compounds is very sensitive to the elemental composition of the humic sample. In a study by Chiou et al. (1987) one natural aquatic humic sample was 4 to 5 times as effective in enhancing solubility than was another; yet, their carbon and oxygen content differed by only 3% and 5% respectively. Grathwohl (1990) observed that organic matter from unweathered shales and high-grade coals had a greater sorption capacity for chlorinated aliphatic hydrocarbons (by more than an order of magnitude) than did organic matter from recent soils, geologically young material, or low-grade coals. The samples exhibiting the greatest sorption capacity had the lowest proportions of oxygen-containing functional groups.

Some authors have attempted to correlate observed partition coefficients with some measurable property of the humic sample; variable results have been reported. The extent of pyrene association with 15 different humic and fulvic acids was found to be positively correlated with the percentage of aromatic carbon in the humic fraction (Gauthier et al., 1987). In contrast, Alberts et al. (1989) observed that for humic substances the aromatic content alone could not explain the partitioning of PAHs. However, it is noted that the aromatic content of humic materials is itself the subject of debate (Farmer & Pisaniello, 1985; Schnitzer & Farmer, 1985; Leenheer et al., 1987; Malcolm, 1990).

Enfield et al. (1989) proposed that the sorption of very hydrophobic solutes to solids with a very low carbon content is not simply dependent on the carbon concentration in general, but rather on the "quality" of the carbon, i.e. aromaticity and lipophilic substituents.

This is obviously a complicated issue and it is possible that the use of base-extracted humic samples for studying the sorption of nonpolar compounds is responsible for some of the variability reported in the literature. At present it is not known to what extent humic material is affected by extraction and clean-up procedures; the chemical structure of humus may be fragile and easily disrupted (Caron & Suffet, 1989). Indeed, Gregor and Powell (1987) observed changes in molecular size distribution (by gel permeation chromatography), equivalent weight, acid dissociation constants and metal binding strengths on subjecting soil fulvic acids extracted by a mild XAD-7/acid-pyrophosphate method (Gregor & Powell, 1986a) to the high and low pH conditions normally encountered during fulvic acid extraction.

Further, aquatic humic substances are operationally defined as the organic matter fraction which is adsorbed on XAD-8 resin at pH 2 and desorbed at pH 13 (Thurman, 1985). There are trace amounts of hydrophobic "humic acids" which are not desorbed from the resin even at pH 13 (Thurman & Malcolm, 1979; Chapter 5); these hydrophobic moieties may also be involved in solubilizing lipid-soluble substrates.

In a preliminary study of this subject, Chiou et al. (1987) observed that Suwannee River humic and fulvic acids enhanced the water solubility of nonionic solutes to the same extent as did the whole, unfractionated water sample. This may imply that the isolation procedure had a negligible effect on the affinity of these humic substances for lipid-soluble compounds. However, the Suwannee River humic and fulvic acids do not have a large capacity for nonpolar substrates; indeed, Suwannee River fulvic acid may not associate with DDT at all (Carter & Suffet, 1982). Hence, any changes in the affinity of humic substances for hydrophobic compounds which may be attributable to isolation procedures are unlikely to be very apparent with the Suwannee samples. Obviously further investigations are required.

1.9.2 Effect of Polarity of the Humic Sample

There is consensus in the literature that nonpolar humic moieties are responsible for the sorption of hydrophobic substrates. For example, the importance of lipoidal material in the sorption of DDT by humic acid has been illustrated by Pierce et al. (1974). Humic acid was Soxhlet extracted with benzene-methanol to remove "free" lipids; the residue was then hydrolyzed with 6 mol L^{-1} HCl (90°C) followed by further benzene-methanol extraction in an attempt to disrupt the humic molecules and remove lipoidal components more tightly bound within the humic structure. The lipoidal fraction was found to have the greatest sorption capacity for DDT, which indicated that the DDT-humic acid interaction is hydrophobic in nature. Also, hexachloro-1,3-butadiene has been reported to be preferentially sorbed to humics rich in hydrophobic paraffinic components (Gauthier et al., 1987).

The interaction of specific fractions of soil and sedimentary organic matter (lipids, humic and fulvic acids, sedimentary organic matter- both lipids and humin) with organic liquids (cresol, aniline, nitrobenzene, and benzene) has been studied by Antworth et al.

(1989). The results indicated that only sorption onto the lipid fraction (material removed by a methylene chloride - methanol (2:1) extraction) is governed by true non-specific hydrophobic partitioning.

The sorption of nonpolar hydrophobic solutes by soil and sedimentary organic matter and its lipid and humin fractions was correlated with the electronic polarizability of the solute (Collazo-Lopez et al., 1989). Humic acid, due to its polar and ionic character, had very little affinity for a series of nonpolar organic solutes. Collazo-Lopez et al. (1989) proposed that it is the free, solvent-extractable (dichloromethane:methanol, 2:1) fraction of soil and sedimentary lipids that dominate the sorption of nonpolar organic solutes.

Humin may represent up to 80% of the total organic carbon in modern sediments; thus, it may exert a considerable influence on the mobility and fate of pollutants in soils and natural waters (Rice & MacCarthy, 1989a). However, the affinity of humin for hydrophobic organic compounds has not yet been extensively studied.

Soil organic matter as a whole has about twice the capacity of the isolated humic acid fraction for the sorption of nonpolar organic substrates (Chiou et al., 1986, 1988). This implies that the overall polarity of soil organic matter is lower than that of soil humic acid; this may be ascribed to the relatively large humin content relative to the more polar humic and fulvic acids (Stevenson, 1982). Differences in molecular size of the various humic fractions may not be such a critical factor as their polarity in effecting partition interactions with nonionic solutes (Chiou et al., 1986, 1987). For example, Garbarini and Lion (1986) reported humin to be approximately three times as effective as soil humic acid in sorption of TCE and toluene. Further, when normalized for organic carbon content, humin exhibited the highest affinity for hydrophobic solutes of all the organic matter fractions studied.

1.9.3 Effect of Molecular Size

The higher molecular weight components of humic matter may be primarily involved in sorption of hydrophobic compounds. By use of ultrafiltration, Whitehouse (1985) demonstrated that PAHs interact mainly with the higher molecular weight organic fraction; Alberts et al. (1989) reached a similar conclusion (also by use of ultrafiltration). Further, the

higher molecular weight components of humic acid have the greatest inhibitory effect on the activity of benzo(a)pyrene (BaP) (Sato et al., 1987).

A certain 'threshold' molecular size is a prerequisite for solubilization of hydrophobic substrates. For example, the water solubility enhancement of DDT by the low molecular weight phenylethanoic acid is quite small on a unit weight basis of the polymer. This observation illustrates the inability of a small organic molecule to effectively promote a partition-like interaction, regardless of its polarity (Kile & Chiou, 1989a). However, Kile and Chiou (1989a) proposed that the greater affinity of high molecular weight humic moieties for hydrophobic compounds is not due to their larger molecular size *per se*, but rather to their lower polarity (fewer polar groups per unit weight (Dell'Agnola & Ferrari, 1971; Collins et al., 1986; Kim et al., 1990)).

Although the presence of large molecules in the humic sample may be a prerequisite for the sorption of hydrophobic compounds, other structures may also be important. For example, the water solubility enhancement of hydrophobic organic compounds by Suwannee River humic and fulvic acids was similar (Kile et al., 1989) even though these humic substances have quite different molecular weights. The molecular weight of Suwannee River fulvic acid is approximately 800 Dalton (Aiken et al., 1989); whereas that of aquatic humic acid is in the range 1 000 to 10 000 Dalton (Thurman et al., 1982). This suggests that the molecular size of humic molecules is a controlling factor for solubility enhancement of nonpolar compounds only up to a certain point, beyond which other factors, such as polarity, predominate (Kile et al., 1989).

Interestingly, "particulate" organic matter (5-300 μm) is not reported to have a greater capacity to sorb hydrophobic compounds than does "dissolved" organic matter (Carter and Suffet, 1982; Garbarini & Lion, 1986).

1.9.4 Effect of pH

Very little systematic work has been carried out on the influence of pH on the ability of humic substances to solubilize nonionic compounds. The water solubility enhancement of DDT and two PCBs by an aquatic fulvic acid was found to be 3.2 times less at pH 8.5 (Kile &

Chiou, 1989a) than at pH 4.0 to 6.5 (Chiou et al., 1986); for soil humic acid the factor was 1.2. Carter and Suffet (1982) observed a decrease in the binding constant of DDT with dissolved humic acid on raising the pH from 6.0 to 9.2.

Perhaps the molecular size fractionation of soil humic acid which occurs with pH (Chapter 5) is a critical factor in pH-dependent hydrophobic solubilization effects.

1.9.5 Reversibility of Humic Substance - Hydrophobic Substrate Interactions

No generalizations can be made about the reversibility of humic substance interactions with hydrophobic compounds. Varying reports exist in the literature; the extent of reversibility is probably compound dependent.

Chiou et al. (1986) obtained about 95% recovery of nonionic compounds from humic substances on extraction with hexane. Also, BaP equilibrated with humic acid was completely released on ultrasonication (20 min) in ethylacetate (Sato et al., 1987). In contrast, Johnsen (1987) reported that the recovery of PAHs (*via* cyclohexane extraction) from natural aquatic humic substances is a function of contact time. Recoveries decreased with increasing contact time and with increasing K_{ow}, or decreasing water solubility, of the PAHs. A similar result was obtained by Carlberg and Martinsen (1982) for recovery of alkanes, PAHs and chlorinated hydrocarbons from aquatic humus. However, considerable variation in recoveries was observed between classes of compounds, and between compounds of the same class.

Perhaps a more pertinent question which should be addressed is the stability of humic-bound hydrohobic compounds. That is, could pollutants apparently detoxified by association with humic substances represent a delayed biological hazard? This aspect has yet to be comprehensively investigated. Recently, Dec et al. (1990) have reported that humic bound 2,4-dichlorophenol is not released on incubation with microorganisms.

1.9.6 Use of Commercial 'Humic' Substances

Although many studies on the interaction of humic substances with hydrophobic substrates have utilized commercial 'humic' samples, it has been well documented that such materials have significantly different composition and structural features than those of natural organic matter (Malcolm & MacCarthy, 1986; MacCarthy & Malcolm, 1989). The use of commercial 'humic' samples is likely to lead to a gross overestimation of the impact of natural dissolved humic material on the solubility and toxicity of nonionic compounds (Kile & Chiou, 1989a; Oris et al., 1990). For example, the water solubility enhancement of DDT and PCBs by natural dissolved organic matter has been compared with that for two commercial 'humic' acids (Aldrich and Fluka) (Chiou et al., 1987). The K_{OC} values obtained with the commercial samples were approximately 3 times larger than those for soil-derived humic acid, about 4 times larger than those of a river humic extract, and approximately 20 times greater than those of acidic water samples and Suwannee River humic and fulvic acids (Kile & Chiou, 1989a). These greater partition coefficients have been attributed to the low carboxyl and carbohydrate contents of the commercial 'humic' acid samples; this may make the molecules more effective in enhancing the water solubility of sparingly soluble organic solutes. Commercial samples may also be of higher molecular weight and may contain some unique molecular structures (Kile & Chiou, 1989a).

Due to the complex nature of humic substances it is important that standard samples are available to workers to allow direct comparison of results. Further, such reference materials should be as representative of the natural substances as possible. A series of such humic substances is available from the International Humic Substances Society (IHSS) (MacCarthy, 1976; Malcolm, 1976) (one of which, Summit Hill humic acid, was used in the present work).

1.9.7 Conclusions

The environmental situation is very complicated. The extent of association between hydrophobic compounds and humic substances will be dependent on the overall particle size, shape, and surface charge characteristics of the humic moieties (Alberts et al., 1989).

Further, these parameters will be governed to some extent by the pH and ionic strength of natural waters or soils; hence, the interactions of nonpolar contaminants with humic substances may vary as water characteristics alter in response to environmental changes. Indeed, Chiou et al. (1987) suggested that the actual elemental composition of humic materials may be influenced by many interrelated environmental factors.

In a recent study on the partitioning of hydrophobic organic compounds in lake water, Eadie et al. (1990) concluded "that we are far from understanding the multitude of processes controlling phase partitioning and subsequent bioavailability". Obviously further investigations on the factors which affect the composition and structure of humic materials are required to enable better predictions of the extent of water solubility enhancement of nonionic pollutants in soils and natural waters.

1.10 MODELS FOR THE SORPTION OF HYDROPHOBIC COMPOUNDS BY HUMIC SUBSTANCES

1.10.1 Partition *Versus* Adsorption Models

There is debate in the literature as to whether the sequestering of nonpolar organic contaminants by humic substances involves surface sorption onto the humic phase or dissolution into hydrophobic humic moieties (MacIntyre et al., 1984; Mingelgrin & Gerstl, 1983). The exact chemical structure of humic materials is also the subject of contention. Proposed models range from cross-linked, predominantly aromatic, high molecular weight polymers (Ogner & Schnitzer, 1971) to a more recent micelle model (Wershaw, 1986) (Section 1.6). Study on the interaction of humic acid with hydrophobic substrates may further elucidate the structure and function of humic acid in soils and natural waters.

The inverse relationship between water solubility of an organic substrate and its 'sorption' by humic substances has been explained from two different viewpoints (Thurman, 1985). Firstly, according to the entropy model, the driving force for association is the hydrophobic effect (the unfavourable orientation of water molecules around a hydrophobic insoluble organic molecule (Tanford, 1973)). The magnitude of this effect is inversely related

to the water solubility of the organic substrate. This entropy model relates only to the organic solute and the solvent (water) and not to hydrophobic bonding to humic moieties.

In contrast, a partition model considers the solubility of the hydrophobic substrate in the organic phase (the humic substance) to be the driving force for humate-nonpolar organic compound associations.

In relation to the structure of humic substances, early models proposed that compounds such as alkanes, fatty acids, amino acids and peptides would be sorbed on the external surfaces and internal voids of humic polymers (Ogner & Schnitzer, 1971). More recently, the sorption of hydrophobic compounds by humic substances has been effectively described in terms of solute partitioning between the aqueous solution and the sorbent organic matter (e.g, Chiou et al., 1986). If one considers humic acid to exist as micelle-like aggregates (Tschapek et al., 1981; Hayase & Tsubota, 1983; Wershaw, 1986) an understanding of this type of mechanism is facilitated. Nonpolar compounds may partition into the hydrophobic core of such structures (Sections 1.6.4 and 1.10.2) in a manner which is mechanistically similar to the solubilization of nonpolar solutes by micelles which form as a microscopic hydrophobic phase through aggregation of amphiphilic monomers (Perdue, 1983; Chiou et al., 1986). That is, interactions between nonpolar solutes and macromolecules are governed primarily by van der Waals forces, with the effectiveness of the partition phenomenon being a function of the aforementioned properties of the humic macromolecules.

Entropy effects are also important. When a hydrophobic molecule is present in aqueous solution an envelope of atypically ordered water molecules forms around it. On contact with a lipohilic phase, this water shell disintegrates and the hydrophobic molecule readily dissolves in the organic solvent. Analogous to a proposed mechanism for the bioconcentration of lipid-soluble compounds (Connell, 1988), the entropy change for this process may provide the driving force for dissolution of nonpolar substrates in a hydrophobic humic phase.

It is likely that the proposed humic acid micellar aggregates also contain some reasonably hydrophilic zones (comprised of hydrogen-bonded polar groups) which extend into the hydrophobic core. Indeed, Chiou et al. (1988) considered soil humic acid to be a

reasonably polar partition medium. Isotherms for the vapour sorption of a range of organic liquids on soil humic acid were linear over a wide range of relative pressure, characteristic of partitioning (or dissolution) of the organic compounds in humic acid. Polar liquids, such as water and ethanol, exhibited markedly greater sorption capacities than did nonpolar liquids, such as hexane and carbon tetrachloride (Chiou et al., 1988).

The following observations are cited as evidence for the sorptive mechanism for solute partitioning into the humic phase (Chiou et al., 1985; Smith et al., 1988): (i) the positive correlation between the humic content of a soil and the uptake of nonionic compounds; (ii) linearity of "soil isotherms" up to high relative concentrations; (iii) low "equilibrium heat effect"; and, (iv) lack of apparent solute competition.

According to some authors, the evidence presented by Chiou and coworkers may not establish a solute partitioning model to the exclusion of a physical adsorption model. For example, linearity of adsorption isotherms and absence of competitive effects are also consistent with a physical adsorption model in which each of the soil minerals and organic phases is considered as a separate adsorbent, thus providing a continuum of adsorption sites (MacIntyre et al., 1984). Further, Mingelgrin and Gerstl (1983) did not consider that inverse correlations between water solubilities and uptake by soil are sufficient proof for a partition process. They suggested that it may not be possible to distinguish between the processes of adsorption and partition. In support of this, they noted that, at low surface concentrations, adsorption isotherms are often linear; similarly, partition of a solute between two solvents is constant only at sufficiently low concentrations. Chiou's use of thermodynamic data to differentiate between partition and adsorption also was questioned by Mingelgrin and Gerstl (1983). Since ΔH and ΔS may vary greatly in magnitude and sign in both these processes, the value of these parameters does not prove or disprove the adsorption or partition models.

In response to these criticisms, Chiou and coworkers (MacIntyre et al., 1984) considered the interpretations of the isotherm linearity and its implication for sorptive mechanisms given by MacIntyre and Smith (MacIntyre et al.,1984) and Mingelgrin and Gerstl (1983) to be misleading. Chiou and coworkers claimed that the linearity should be evaluated in terms of the relative saturation (the ratio of equilibrium concentration to

solubility) rather than the absolute concentration of the solute in the medium from which it is sorbed. High saturations for sparingly soluble solutes will be reached when equilibrium concentrations are close to solubility limits, even though absolute concentrations are low. On saturation of the aqueous phase, the humic phase in equilibrium with it must simultaneously reach saturation. Chiou and coworkers have demonstrated that the sorption of organic compounds from water onto soil exhibits linear isotherms to high relative concentrations and with no competition between solutes, thus supporting a partition process. In contrast, when sorption was controlled by adsorption on soil minerals, distinctive curvature and competitive effects were evident even at very low relative concentrations (Chiou et al., 1981; Chiou & Shoup, 1985).

Recently, Chiou et al. (1990) have established that soil organic matter has a relatively low suface area for adsorption. Therefore, "it is no longer tenable to ascribe the sorption of organic pollutants and pesticides by soil organic matter to a high surface area that it in fact does not possess" (Chiou et al., 1990).

1.10.2 Application of the Micelle Model for Humic Acid

A micelle model for humic acid was invoked by Abdul et al. (1990) to explain the enhanced removal of hydrophobic contaminants from hydrogeologic systems in the presence of humic acid. Boehm and Quinn (1973) also proposed a micelle model for humic substances to explain the solubilization of hydrocarbons in seawater. Solubilized hydrocarbons were reported to exist in a "semicolloidal or micellar state formed by interactions [with] humic-like monomers in solution".

Humic acid may have a relatively high critical micelle concentration (cmc). Estimates are: 18 g L^{-1} (Piret et al., 1960), 1 g L^{-1} (Hayano et al., 1982), and 1- 10 g L^{-1} (Hayase & Tsubota, 1983). Therefore, micelle formation and hydrocarbon solubilization might not be expected to occur in seawater (DOC = 0.3 to 20 mg L^{-1}). However, the addition of salts decreases the cmc and increases micellar size and weight. In agreement with this, Carter and Suffet (1982) have observed that high ionic strength enhances sorption of hydrophobic

compounds by humic substances. The addition of solubilizates (e.g. hydrocarbons) also lowers the cmc (Elworthy et al., 1968).

Humic acids extracted from marine sediment increased the water solubility of hydrocarbons (Shinozuka et al., 1987). The solubility of n-alkanes and PAHs increased sharply with humic acid concentration below 0.01%, levelled off between 0.01 to 0.1% and markedly increased again above 0.1%. Shinozuka et al. (1987) proposed that at low humic acid concentrations, hydrocarbons are adsorbed on the hydrophobic moieties of humic acid; whereas above 0.1% "hydrocarbon molecules are solubilized into humic acid aggregates like the solubilization of water insoluble substances into surfactant micelles". In support of this, Hayano et al. (1982) reported that humic acids from marine sediments form aggregates above a concentration of 0.1% (1 mg mL^{-1}).

The enhanced water solubility of nonionic organic compounds in the presence of humic substances is evidence for humic-hydrophobic solute interactions (Chiou et al., 1986). A cosolute can produce an enhancing effect on solute solubility either by changing the solvency of the medium or by direct solute interaction (either by adsorption or by partitioning). According to Chiou et al. (1986), humic substances at low (environmentally significant) concentrations are unlikely to have a strong impact on water solvency; it is also questionable that their polar functional groups would exhibit specific interactions with nonionic organic substrates in aqueous solutions as the hydrophilic functional groups of humic molecules will be more favourably associated with water.

Chiou et al. (1986) proposed (on the basis of molecular weight and elemental analysis data) that soil humic acids do provide a sufficiently large intramolecular nonpolar environment for promoting a partition-like interaction with relatively hydrophobic organic solutes. This interaction was suggested to be "mechanistically similar to the solubilization effect of relatively water-insoluble organic solutes by micelles in which a microscopic organic phase is formed through aggregation of surfactant monomers". However, it is noted that the concentration range over which humic acid aggregates (1 to 10 g L^{-1}) is approximately 1 000 times the typical environmental humic substance concentration (0.5 to 10 mg L^{-1}; Thurman, 1985). Further, fulvic acid is the predominant soluble humic fraction in natural waters.

(However, Roemelt & Seitz (1982) proposed a micelle model to explain binding of perylene by fulvic acid.)

The surface tension versus solubility plot for Suwannee River fulvic acid, up to 30 000 ppm, shows no inflexion that would be indicative of micelle formation (Chiou, pers. comm., 1989). Yet, conventional methods for cmc determination (such as changes in surface tension with surfactant concentration) are much less sensitive to incipient formation of aggregates than are measurements of water solubility enhancement of nonpolar compounds. Any monomer-micelle transition over a concentration range should give rise to a very sharp change in water solubility enhancement. This was not observed for humic and fulvic acids up to 100 ppm (Chiou et al., 1986).

It is possible that, due to the heterogeneous nature of humic substances, a sharp transition may not be observed in the water solubility enhancement of nonpolar compounds with increasing humic concentration. Precedent for a relatively diffuse cmc is provided by a study of the water solubility enhancement of nonpolar compounds by nonhomogeneous synthetic surfactants (the Triton series and Brij 35; Kile & Chiou, 1989b). It is important to note that with these high molecular weight synthetic surfactants no significant change is observed in the water solubility enhancement effect until surfactant concentrations exceed about 100 ppm. This value is the highest concentration of humic substance studied by Chiou et al. (1986). It would be of theoretical interest to investigate whether any change in solubility enhancement by humic substances is exhibited at concentrations greater than 100 ppm (although this would not be environmentally relevant).

The cmc is given by the concentration at which a sharp break in the slope of the surface tension versus log(surfactant concentration) plot occurs. Kile and Chiou (1989b) observed that the cmc for molecularly homogeneous surfactants is quite well defined, but the monomer-micelle transition zone becomes considerably less sharp for nonhomogeneous surfactants. The lack of a well defined cmc is proposed to result from the successive micellization of heterogeneous monomers at different stoichiometric concentrations of the surfactant (due to their different structures and different solubilities in water), which results in a breadth of the monomer-micelle transition zone. The shape of the water solubility

enhancement plots near the cmc for heterogeneous synthetic surfactants was indicative of a continuum of aggregate formation. It is possible that a similar mechanism is applicable to the solubilization of hydrophobic compounds by humic substances. Humic acid is heterogeneous, and successive solubilization of different molecular size moieties on altering the pH has been observed (Chapter 5). However, a linear dependence of apparent solute solubility on the concentration of humic substance was observed (Chiou et al., 1986), whereas a nonlinear relationship held for synthetic surfactants at concentrations below the cmc (Kile & Chiou, 1989b).

The synthetic surfactants studied by Kile and Chiou (1989b) were nonionic; micelles of ionic surfactants (such as humic acid) are expected to be more strongly hydrated at the charged sites, and such hydration may restrict access to portions of the inner hydrophobic core, thus reducing the partition efficiency of the solute. Comparison of the solute partition coefficients for synthetic surfactants with those for dissolved aquatic humic and fulvic acids showed that the coefficients for humic substances were comparable in magnitude with those for monomeric nonionic surfactants. However, they were orders of magnitude lower than values for either ionic or nonionic micelles.

In conclusion, aquatic humic substances at typical environmental concentrations (< 100 mg L^{-1}) do not form the kind of micellar environment that is established by synthetic surfactants (Kile & Chiou, 1989b).

1.11 SCOPE OF THIS WORK

In this work, the solubility and aggregation properties of humic and fulvic acids, and their interactions with hydrophobic metal complexes and with aqueous metal ions were investigated.

As discussed above, humic substances are heterogeneous macromolecules whose composition is poorly characterized. Hence, a quantitative interpretation of such complex systems is not feasible. Therefore, in this work a 'comparative' approach was adopted in which data for different humic samples, or for the same sample measured under different conditions, were compared directly. Calculation of 'absolute' parameters was not attempted.

Humic substances 'interfere' with the application or interpretation of many experimental techniques; for example, they adsorb on electrode surfaces. These effects were studied, and experimental conditions were developed to minimize or control such interferences.

The solubility and aggregation properties of humic acid are fundamental to an understanding of the macromolecular structure of humic substances, of their mobility in soils and natural waters, and are relevant to the development of extraction protocols. These were studied as a function of pH and ionic strength *via* gel permeation chromatography and equilibrium dialysis. It was observed that predominantly smaller humic acid molecules were soluble below pH 4; the solubility of the larger molecules increased with increased pH and with decreased ionic strength.

Extraction of humic acids from soils has traditionally involved the use of strongly alkaline solutions with the concomitant risk of alteration of the humic material and coextraction of 'impurities', followed by acid precipitation. In contrast, aquatic humic substances are isolated by concentration on macroporous XAD resins. XAD resins have been used to isolate fulvic acids from soil extracts; a low-ash fulvic sample is obtained (Gregor & Powell, 1986a). Application of XAD resins to the isolation, and purification, of humic acids from soil extracts was investigated in this work. Results indicated that these adsorbents would not be suitable for this purpose: large humic acid molecules were excluded

from the XAD resins, and the humic acid components which were adsorbed could not be completely desorbed at pH 7 (or even pH 11). These studies highlighted the operational nature of the fraction defined as 'humic acid' and raised questions about the reported differences between aquatic and soil-derived humic substances; such apparent differences could result in part from the different extraction procedures used.

Hydrophobic compounds are toxic to biota and there is an extensive literature on the interaction of these species with humic substances and the concomitant amelioration of toxicity to biota. Recently, it has been observed that hydrophobic copper(II) complexes are extremely toxic to algae. In this work, the interaction of humic substances with hydrophobic metal complexes was studied *via* ASV (at a chemically modified TMFE), solvent extraction, equilibrium dialysis (using radiotracers and electrochemical detection), visible absorption spectroscopy, and cathodic stripping voltammetry. Only Cu(II) complexes were found to be stable enough to exist at typical environmental concentrations. It was established that humic substances (HS) could displace a low molecular weight ligand (L = 8-hydroxyquinoline or 1-(2-pyridylazo)-2-naphthol) resulting in formation of a ternary complex, HS-Cu-L. Algal assays demonstrated that humic acid could thus ameliorate the toxicity of hydrophobic copper(II) complexes, but only if the displaced ligand itself is not toxic.

Humic substances are the ligands dominating complexation of metal ions in the environment. Indeed, the metal buffering action provided by humic substances is essential to sustain life in natural systems (Buffle et al., 1990a,b). Factors which determine the bioavailability of metal ions include the lability and stability of their complexes with humic substances. In this work the interaction of humic and fulvic acids with divalent metal ions (Cu(II) and Pb(II)) was investigated by anodic stripping voltammetry (ASV) and ion selective electrode (ISE) potentiometry. ASV probed the relative lability of metal complexes, while ISE potentiometry provided a measure of their stability.

ASV studies focussed on identifying, and subsequently minimizing, the effects of adsorption of humic substances on the electrode surfaces (a hanging mercury drop electrode (HMDE) and a thin mercury film electrode (TMFE)). Recently, hydrophilic polymer coatings

(Nafion) have been reported to minimize electrode fouling by organic compounds. In this work, the properties of Nafion coated electrodes (TMFE and bare glassy carbon) were investigated in the absence and presence of humic substances. Some studies on the properties of a TMFE were also performed. Although less reproducible, ASV studies using a bare glassy carbon electrode were feasible in mildly acidic solutions (pH 4.8).

Both humic and fulvic acids readily complex with metal ions. The humic fraction which forms the most stable complexes has not been unequivocally established. Fulvic acids are the predominant dissolved humic fraction in natural waters, whereas humic acids are more likely to be associated with the particulate fraction, e.g. iron oxide colloids. Hence studies on the relative stability of humic and fulvic acid complexes are important to further elucidate the speciation of metals in the environment. The amount of metal ions able to be complexed by humic substances (the complexation capacity) is also important. For example, this parameter may give an indication of the ability of a natural system to cope with an influx of metal ions (as may result from atmospheric acidic precipitation).

In this work ISE potentiometry was used to probe the 'complexation capacity' and stability of Cu(II) complexes with humic substances. The complexation capacity of humic acid for Cu(II) was greater than that for fulvic acid, indicating a more 'efficient' distribution of a smaller number of polar groups into chelating moieties. For both humic and fulvic acids the complexation capacity increased with increased pH (range 5.0 to 7.0). These measurements indicated that the proportion of carboxyl groups involved in Cu(II) complexation was greater for humic acid than for fulvic acid.

Normalized to carboxyl group content, humic acid formed complexes of much greater stability than did fulvic acid. The apparent stability of the Cu(II) complexes decreased with increased metal-to-ligand ratio, and with increased ionic strength. The larger humic acid molecules formed more stable Cu(II) complexes than did the smaller components. The Cu(II) ISE was found to be a useful probe for studying the interaction of humic substances with other metal ions. For example, the Cu(II) binding curves for humic substances were displaced to higher pH (weaker binding) in the presence of Mg(II) and Al(III).

The nature of the binding sites in humic substances responsible for metal ion complexation has been the subject of debate. Frequently, the likely functional groups involved have been inferred from products of chemical degradation analyses, or by comparison of 'stability constants' measured for humic substances with those for discrete ligands. As noted above, these assumptions may not be valid. In this work a more direct approach was adopted. Attempts were made to simulate the Cu(II) binding curves for humic substances by comparison with those calculated or measured for discrete ligands. This allowed humic functional groups most (malonate, citrate) and least (salicylate, phthalate) likely to be involved in Cu(II) chelation to be identified

As outlined above (Section 1.8), the heterogeneous nature of humic substances prevents quantitative analysis of their protonation and metal complexation equilibria. Therefore, to gain expertise in measurement of metal-ligand solution equilibria, several simpler systems were quantitatively investigated. Specifically, the systems studied were: Al(III)-malonic acid, Al(III)-isocitric acid, Cu(II)-5-methoxy-N-(2-hydroxybenzyl)sarcosine (MHBS), and Cu(II)-butane-1,2,3,4-tetrabutanoic acid. Both Al(III) and Cu(II) are important metal ions in soil solutions and natural waters; the ligands chosen are consistent with structures likely to be present in humic substances. Study of the Al(III)-isocitrate system further elucidated the coordination of citrate with Al(III) (which forms an exceptionally stable complex). The Cu(II)-MHBS system illustrated the phenomenon of 'cascade binding' (enhanced intramolecular coordination of weak donor groups by virtue of their proximity to strongly coordinating sites) which may contribute to metal ion complexation by humic substances.

CHAPTER 2

PROCEDURE FOR THE DETERMINATION OF STABILITY CONSTANTS BY POTENTIOMETRIC TITRATION

This chapter describes the methodology used for the determination of stability constants from potentiometric titration data. It is divided into 2 sections. Section A describes the nonlinear least squares procedure used to calculate stability constants from the titration data. Section B outlines the procedure and theory for the calibration of a glass electrode as a hydrogen ion concentration probe.

SECTION A: CALCULATION OF STABILITY CONSTANTS FROM POTENTIOMETRIC TITRATION DATA

2.1 Ligand Protonation Equilibria

Titration of a weak acid with a strong base generates a series of deprotonated species described by the following equilibria and protonation constants:

$$H + H_{n-1}L \leftrightharpoons H_nL \qquad K_{nH} = \frac{[H_nL]}{[H_{n-1}L][H]}$$

Charges have been omitted for clarity.

The total concentration of ligand (TL) and of titratable protons (TH) in solution can be expressed, respectively, in terms of the equilibrium concentrations of all ligand-containing species and all proton-containing species, *viz:*

$$TL = [L] + [HL] + \ldots + [H_nL]$$

$$TH = [H] + [HL] + \ldots + n[H_nL] - [OH]$$

The term [OH] arises from hydrolysis reactions of the type:

$$H_{n-1}L + H_2O \leftrightharpoons H_nL + OH$$

TL and TH were derived, respectively, from the initial concentration of ligand and the initial concentration of titratable protons. These experimentally determined quantities were corrected for dilution, for the concentration of added acid, and for the concentration of added alkali.

The secondary concentration variable, $\bar{n}_H$, was used to calculate protonation constants from the potentiometric titration data. $\bar{n}_H$ describes the degree of protonation of a ligand and is defined by:

$$\bar{n}_H(obs) = (TH - [H] + [OH])/TL$$

Hence, $\bar{n}_H$ can be calculated from the experimentally determined values for TH, [H], [OH], and TL at each datum point in the titration. This value is referred to as $\bar{n}_H(obs)$.

$\bar{n}_H$ can also be expressed as a function of the protonation constants of the ligand and of the equilibrium hydrogen ion concentration. This value of $\bar{n}_H$ is referred to as $\bar{n}_H(calc)$:

$$\bar{n}_H(calc) = \frac{K_{1H}[H] + 2K_{1H}K_{2H}[H]^2 + \ldots}{1.0 + K_{1H}[H] + K_{1H}K_{2H}[H]^2 + \ldots}$$

By assuming values for the protonation constants, $\bar{n}_H(calc)$ can be determined at each datum point. The protonation constants are treated as variable parameters, and a nonlinear least squares analysis is performed which minimizes the function $\Sigma(\bar{n}_H(obs) - \bar{n}_H(calc))^2$ over all datum points.

Consider the protonation equilibria of the diprotic ligand malonic acid as a specific example. The protonation equilibria are given by:

$$H + L \leftrightharpoons HL \qquad K_{1H} = \frac{[HL]}{[H][L]}$$

$$H + HL \leftrightharpoons H_2L \qquad K_{2H} = \frac{[H_2L]}{[HL][L]}$$

The mass balance equations for TH and TL are:

$$TH = [H] + [HL] + 2[H_2L] - [OH].$$

$$TL = [L] + [HL] + [H_2L].$$

Hence, $\bar{n}_H(obs) = (TH - [H] + [OH])/TL$.

These equations can also be expressed in terms of the protonation constants:

$$TH = [H] + K_{1H}[H][L] + 2K_{1H}K_{2H}[H]^2[L] - [OH].$$

$$TL = [L] + K_{1H}[H][L] + K_{1H}K_{2H}[H]^2[L].$$

Hence, an expression for $\bar{n}_H(calc)$ is obtained:

$$\bar{n}_H(calc) = \frac{K_{1H}[H] + 2K_{1H}K_{2H}[H]^2}{1.0 + K_{1H}[H] + K_{1H}K_{2H}[H]^2}$$

In some situations, for example when the ligand salt is hydrated, the value of TL may not be well defined. In such a case it is desirable to have TL as a variable parameter. This requires the function $\Sigma(TH_{obs} - TH_{calc})^2$ to be minimized over all datum points, where:

$$TH_{obs} = [H] + [HL] + \ldots + n[H_nL] - [OH]$$

$$TH_{calc} = \frac{[TL](K_{1H}[H] + 2K_{1H}K_{2H}[H]^2 + \ldots)}{1.0 + K_{1H}[H] + K_{1H}K_{2H}[H]^2 + \ldots}$$

2.1.1 Ionic Strength Corrections

A thermodynamic protonation constant (K^{th}_{nH}) is expressed as a function of activities ({}):

$$K^{th}_{nH} = \frac{\{H_nL\}}{\{H\}\{H_{n-1}L\}}$$

The activity coefficient (γ) relates the activity of each species to its concentration:

$$K^{th}_{nH} = \frac{[H_nL]}{[H][H_{n-1}L]} \frac{\gamma_{HnL}}{\gamma_H\gamma_L}$$

The product of concentrations is defined as a 'concentration constant' (stability constant), K_{nH}. The value of K_{nH} is dependent on the ionic strength and will have a constant value only if the ionic strength of the medium is constant. For potentiometric titrations performed with a background electrolyte concentration of only 0.10 mol L^{-1} the assumption of constant ionic strength throughout the titration may not be valid and could lead to significant errors (May et al., 1985). That is, the formation or consumption of polyvalent ions will generate small but significant changes in ionic strength. For example, in the titration of a 5 x 10^{-3} mol L^{-1} solution of the tetraprotic ligand butane-1,2,3,4-tetracarboxylic acid, the ionic strength will increase by 0.045 as ñ changes from 4.0 to 0.0. Hence, it is necessary in the refinement process to 'correct' the stability constants (trial parameters) to the appropriate ionic strength at each datum point. In the present work the expected change in ionic strength due to complex formation was calculated for each titration. This effect was taken into account by preparing the titration solutions so that the ionic strength at the mid-point of the titration would be 0.10 mol L^{-1}. Typically the range of ionic strengths was 0.0825 - 0.1275 mol L^{-1}. The effect of these small changes in ionic strength on the calculation of stability constants was assessed by use of the equation:

$$\log K' = \log K^{th} \frac{\gamma_H\gamma_L}{\gamma_{HL}}$$

Log K', which is the parameter being refined, varies with ionic strength, i.e. for each datum point. The Debye-Hückel equation was used to relate the value at I = 0.100 mol L^{-1} (K_i) to the value at any other ionic strength (K_i'):

$K_i' = K_i.10^a$, where $a = \frac{Az^2\sqrt{I}}{1+\sqrt{I}} - \frac{Az^2\sqrt{0.1}}{1+\sqrt{0.1}}$ and $I = \frac{1}{2}\Sigma c_i z_i^2$ (c_i is the concentration of ion i; z is the valency of ion i). The value used for A was 0.5115 (Robinson & Stokes, 1959).

A specific example of this ionic strength correction is now given. Consider titration of the tetraprotic ligand butane-1,2,3,4-tetracarboxylic acid. The value of K_{nH} at the actual ionic strength of the medium is equal to the value of K_{nH} at an ionic strength of 0.100 mol L^{-1} multiplied by the Debye-Hückel term:

For the reaction: $H^+ + L^{4-} \leftrightharpoons HL^{3-}$

$$\log K'_{1H} = \log K^{th}_{1H} + \log\gamma_H + \log\gamma_{L^{4-}} - \log\gamma_{HL^{3-}}$$

$$= \log K^{th}_{1H} - \frac{A\sqrt{I}}{1+\sqrt{I}} - \frac{16A\sqrt{I}}{1+\sqrt{I}} + \frac{9A\sqrt{I}}{1+\sqrt{I}}$$

$$= \log K^{th}_{1H} - \frac{4.092\sqrt{I}}{1+\sqrt{I}}$$

The actual ionic strength, AI, is defined as: $[KCl] + [H_3L] + 3[H_2L] + 6[HL] + 10[L]$. The reference ionic strength, AISTR, is equal to 0.100 mol L^{-1}.

Therefore, $\log K'_{1H} = \log K^{th}_{1H} + \frac{-4.092\sqrt{AI}}{1+\sqrt{AI}} + \frac{4.092\sqrt{AISTR}}{1+\sqrt{AISTR}}$

By a similar process, equations for the ionic strength correction of K_{2H}, K_{3H} and K_{4H} are obtained, *viz:*

$$\log K'_{2H} = \log K^{th}_{2H} + \frac{-3.069\sqrt{AI}}{1+\sqrt{AI}} + \frac{3.069\sqrt{AISTR}}{1+\sqrt{AISTR}}$$

$$\log K'_{3H} = \log K^{th}_{3H} + \frac{-2.046\sqrt{AI}}{1+\sqrt{AI}} + \frac{2.046\sqrt{AISTR}}{1+\sqrt{AISTR}}$$

$$\log K'_{4H} = \log K^{th}_{4H} + \frac{-1.023\sqrt{AI}}{1+\sqrt{AI}} + \frac{1.023\sqrt{AISTR}}{1+\sqrt{AISTR}}$$

These 'corrected' values of log K_{nH} are the parameters which are refined in the nonlinear least squares calculations.

The effect of these small changes in ionic strength on $K_H^{'}$ was found to be insignificant for the concentrations of di- and triprotic ligands used in the present work; for a tetraprotic ligand a small change in the mean value of log K_{4H} was observed.

2.1.2 Ion-Pairing Reactions

By use of the constant ionic medium method in determination of stability constants, interactions between the ligand and ions in the inert electrolyte make the log K_{nH} values dependent on the specific cations and their concentration (Daniele et al., 1985a). If the weak interactions between the (ionized) ligand and the background electrolyte are taken into account, the dependence of the stability constants on the ionic strength (in the range 0 to 1 mol L^{-1}) will be a function only of the charges and concentrations of ions involved in the reaction. Daniele and coworkers have reported the stability constants for complexation of alkali metals by a range of ligands (e.g. Daniele et al., 1983, 1985b).

In the cases where such stability constants had been reported for the ligands studied in the present work, the effect of ion-pairing reactions on the calculated stability constants was assessed. A significant impact was observed. For example, the mean value of log K_{1H} for isocitric acid was increased by 0.20 log units when K^+ ion pairing was included in the model (log K_{2H} and log K_{3H} were not affected). However, there was no significant improvement in the least-squares fit. An improved fit to the data may be expected because inclusion of this parameter shifts the distribution curve for the deprotonated ligand significantly, i.e. it acknowledges that a significant amount of HL is dissociated into K^+L^{2-} as well as L^{2-}.

As a specific example, consider calculation of the protonation constants for malonic acid including the ion pairing reaction to form K^+L^{2-} (log K = 0.68; Daniele et al., 1985b). For a background electrolyte concentration of 0.10 mol L^{-1} (KCl), the equation for $\tilde{n}_H$(calc) is given by:

$$\tilde{n}_H(\text{calc}) = \frac{K_{1H}[H] + 2K_{1H}K_{2H}[H]^2}{1.479 + K_{1H}[H] + K_{1H}K_{2H}[H]^2}$$

where 0.479 is equal to $10^{0.68}[K^+]$.

2.2 Metal-Ligand Equilibria

A thermodynamic equilibrium constant, β^{th}_{pqr}, is expressed as a function of activities ({ }) and is defined by a law of mass action:

$$\beta^{th}_{pqr} = \frac{\{ML\}}{\{M\}\{L\}}$$

Such thermodynamic equilibrium constants apply to solutions of any ionic strength and are functions only of temperature and pressure.

The activity coefficient (γ) relates the activity of each species to its concentration: $\beta_{pqr} = \beta^{th}_{pqr} \frac{\gamma_M \gamma_L}{\gamma_{ML}}$, where β_{pqr} is the concentration quotient defined as the stability constant.

The activity of a species will be equal to its concentration only in infinitely dilute solutions. As the ionic strength increases, ions of opposite charge interact leading to a decrease in their effective activity (Fischer & Peters, 1970). Hence, stability constants which are defined in terms of the concentrations of species are dependent on the ionic strength of the medium, and β_{pqr} will be constant only if the term $\frac{\gamma_M \gamma_L}{\gamma_{ML}}$ is constant under the experimental conditions. Although several equations have been proposed for the estimation of activity coefficients, their values cannot be calculated accurately. However, this problem can be overcome by use of a constant ionic strength medium in which the activity coefficients of all species can be assumed to be constant (Beck, 1970) and hence the 'stability constant' will be numerically constant. In the present work, a constant ionic strength of 0.10 mol L^{-1} (KCl) was used.

In the determination of stability constants for metal-ligand complexes the equilibria which must be considered are:

(i) Protonation of the ligand: $L + nH \leftrightharpoons H_nL$

(ii) Hydrolysis of the metal ion: $xM + yOH \leftrightharpoons M_x(OH)_y$

(iii) Metal-ligand equilibria: $pM + qH + rL \leftrightharpoons M_pH_qL_r \quad (\beta_{pqr})$

A series of mass balance equations can be written for each component:

Total metal (TM) = $[M] + \Sigma p[M_pH_qL_r]$.

Total ligand (TL) = $[L] + \Sigma r[M_pH_qL_r]$.

Total acid (TH) = $[H] + \Sigma q[M_pH_qL_r] - [OH]$; where $[OH] = K_{cw}[H]^{-1}$.

The concentration of OH is calculated; it is dependent on the value assumed for K_{cw} (where $K_{cw} = K_w \frac{a_{H2O}}{\gamma_{H^+}\gamma_{OH^-}}$) For the present work (at 25°C) K_w was 1.007×10^{-14} and the value used for the activity coefficient term was 1.0/0.625 (valid in O.1 mol L^{-1} KCl at 25°C; Harned & Owen, 1958).

Stock metal and ligand solutions were standardized separately (as described in Chapter 3) and the stability constants for the metal-ligand systems were determined by potentiometric titration of a metal-ligand solution with standard alkali (KOH). pH was measured as a function of titre; the equilibrium hydrogen ion concentration was obtained from the measured pH by the procedure described in Section B. Ligand protonation constants were determined from separate titrations.

Graphical methods were used for initial data analysis. Specifically, plots of ZC *versus* pH, and of ñ *versus* pL (Bjerrum plots) were constructed. ZC is defined as the average number of moles of alkali reacted (added) per mole of ligand (= ALK/TL), and ñ is the average number of ligand molecules coordinated per metal ion (=(TL-$\sum_0^n H_nL$/TM). These graphical techniques allowed the terminal equilibrium species to be identified, and approximate values for the relevant stability constants to be obtained. If the value of ZC for a species H_nL exceeds n then this indicates that metal hydroxy species and/or ternary M-L-OH complexes must make a significant contribution to the equilibrium system.

In the absence of ternary species, ñ correctly defines the average number of ligands bound per metal ion and ñ/pL plots for titrations at different TM:TL ratios and concentrations will be superimposable. Any deviation from a coincident family of plots (i.e. a systematic dependence of the ñ/pL plots on TM and TL) indicates the formation of hydroxy and/or polymeric species. For such a case the meaning of ñ is complicated (Avdeef, 1985).

There has been considerable publication on the validity of computational approaches used in various programs to determine stability constants (e.g. Baeza et al., 1989; Leung et al., 1988; Legget, 1985). In the present work stability constants were calculated by use of the FORTRAN program ORGLS (Busing & Levy, 1962). Unlike SUPERQUAD (Gans et al., 1985) or LETAGROPVRID (Ingri & Sillen, 1964), ORGLS is not written for the general case. Hence, separate subroutines were written for each metal-ligand system studied. These subroutines have been reported by Gregor (1987) and were modified in the present work to

allow one of the analytical concentrations to be refined (taking into account the dilution which occurs over the course of the titration). The subroutines set up an array of mass balance equations for each species after each addition of alkali. In the above mass balance equations, the unknown parameters are [M], [L], and β_{pqr}. Calculation of TH_{calc} requires trial values for the parameters (stability constants), and values for the free metal ([M]) and free ligand ([L]) obtained from iterative solutions of the other two equations.

A trial value for [L] for the first (most acidic) datum point was estimated by assuming no complexing had occurred, i.e. $[L] = TL/(1.0 + K_{1H}[H] + K_{1H}K_{2H}[H]^2 + \ldots)$. This trial value for [L] was then used in the mass balance equation for TM to find an approximate value for [M] (by a Newton-Raphson iterative procedure), the approximate value of [M] was then substituted into the equation for TL to find an improved value for [L], also found by Newton-Raphson iteration. This refinement process was continued until the values calculated for log [L] in subsequent cycles did not differ by more than 0.001. The final values of [L] and [M] were then substituted into the mass balance equation for TH to obtain TH_{calc}. For the n^{th} datum point the value of [L] from the $(n-1)^{th}$ point was taken as the initial estimate.

The stability constants which provided the 'best' fit to the data were calculated by a nonlinear least squares procedure which minimized the function: $\sum_{i=1}^{n} w_i(TH_{obs}-TH_{calc})^2$ over all datum points, where TH_{obs} and TH_{calc} denote the experimental and calculated values of the total titratable acidity respectively, and w_i is the statistical weight for the i^{th} datum point. As an example, consider complex formation in the Al(III)-malonic acid system. The following stepwise equilibria were considered:

$$Al^{3+} + L^{2-} \leftrightharpoons AlL^{+} \qquad K_1 = \frac{[AlL^{+}]}{[Al][L]}$$

$$AlL^{+} + L^{2-} \leftrightharpoons AlL_2^{-} \qquad K_2 = \frac{[AlL_2^{-}]}{[AlL^{+}][L]}$$

$$AlL_2^{-} + L^{2-} \leftrightharpoons AlL_3^{3-} \qquad K_3 = \frac{[AlL_3^{3-}]}{[AlL_2^{-}][L]}$$

The ligand protonation equilibria (K_{1H} and K_{2H}) and hydrolysis reactions of the metal ion were also considered. The following mass balance equations were obtained:

$TL = K_{1H}[H][L] + K_{1H}K_{2H}[H]^2[L] + K_1[Al][L] + 2K_2K_1[Al][L]^2 + 3K_3K_2K_1[Al][L]^3$.

$TM = K_1[Al][L] + K_2K_1[Al][L]^2 + K_3K_2K_1[Al][L]^3$.

These equations were solved by the Newton-Raphson iterative procedure described above. The refined value of [L] was then used to calculate TH_{calc}:

$TH_{calc} = K_{1H}[L][H] + 2K_{1H}K_{2H}[L][H]^2$.

(The value of [M] does not appear in this equation for TH_{calc} because no ternary species ($MH_{n-x}L$) were formed in this system.)

This type of computational procedure is based on the assumption that the concentration or activity of one component of the system (either H, M, or L) can be measured. The 'ease' with which the glass electrode can be calibrated to determine hydrogen ion concentration (Section B) makes pH potentiometric titrations the prime method for the determination of stability constants.

The precision of the calculated stability constants and the convergence quality in the least squares process are tests of the validity of the equilibrium model. If it describes the data well, the residuals should be randomly distributed throughout the data set.

The approach used in the present work was to include the minimum number of species in the data analysis; additional species were added only if there was sufficient evidence for their existence and if their inclusion in the analysis significantly improved the fit to the data.

2.2.1 Sources of Error in Stability Constant Determinations

Braibanti et al. (1982) applied a statistical analysis of variance to the values of the stability constants determined for the glycinate-proton and glycinate-nickel(II) systems (which were determined by pH titration in a number of laboratories). For every constant, the differences between titrations were larger than the variance within one titration. Therefore, as recommended by these authors, the equilibrium constants in the present work were refined separately for each titration; the values for different titrations were then averaged (unless otherwise stated).

Meloun et al. (1988) reported that the reliability of stability constants obtained by regression analysis based on potentiometric data is dependent on the calibration of the glass electrode, the algorithm used, and the parameters selected for refinement. Recently, a set of recommended procedures for data collection and evaluation, model selection, assessment of

the effects of ionic strength corrections and of weighting, and the refinement of values has been reported (May & Murray, in press).

2.2.2 Weighting of Data

In a titration some datum points will be more reliable than others (because they are less distorted by experimental errors); hence, it is theoretically possible to improve the information content of the data set by giving greater emphasis where it is due. Indeed, various weighting schemes have been proposed (e.g. Avdeef, 1983; Kateman et al., 1983). However, 'correct' weights cannot yet be evaluated in a rigorous manner and, until the systematic effects of errors can be eliminated, the way in which the data are weighted is largely irrelevant (May & Murray, 1988).

In the present work all datum points were assigned equal statistical weight, but in the region of significant inflexions in the titration curve (end-points) fewer datum points were included in the data set for refinement.

SECTION B: CALIBRATION OF THE GLASS ELECTRODE AS A HYDROGEN ION CONCENTRATION PROBE

Another aspect of this work which involved iterative calculations was calibration of the glass electrode as a hydrogen ion concentration probe.

The concept of pH is unique amongst physicochemical quantities, in that its definition ($-\log_{10}a_H$) involves a single ion activity coefficient which is immeasurable (Covington et al., 1985). The single ion activity coefficient for the hydrogen ion can be calculated (approximately) by use of the Debye-Hückel equation. However, this relationship is not reliable for mixed electrolyte solutions as would be involved in studies on metal-ligand equilibrium systems.

Potentiometric titration, with a glass electrode/calomel electrode pair, was used in the present work for the determination of stability constants (Section A). Solving the mass balance equations for these systems requires knowledge of the equilibrium hydrogen ion *concentration*. However, the glass electrode is assumed to respond to the *activity* of hydrogen ions (Hamer & Acree, 1939). Interpretation of its response either in terms of hydrogen ion

concentration ($[H^+]$), or hydrogen ion activity, involves assumptions associated with liquid junction potentials and single ion activity coefficients. Therefore, a methodology was adopted which compensated for the contribution from liquid junction potentials and allowed $[H^+]$ to be obtained directly from the pH measured by the electrode pair. This is now discussed. (Experimental details are given in Chapter 3.)

The emf of the cell used in the present work is given by:

$E = E^\circ + E_{as} + E_{LJ} - (RT/F)\ln a_{H^+}$; where E° is the standard emf for the reference electrode in saturated KCl, E_{as} is the asymmetry potential of the glass electrode, and E_{LJ} is the liquid junction potential. The pH of an unknown solution (pH') is related to the pH of a standard solution (pH^s) and the measured emf by:

$$pH' = pH^s - \frac{(E'_{LJ} - E^s_{LJ}) + (E^s - E')}{2.303RT/F}$$

Conventionally, standard pH solutions are defined by the NBS primary buffers (Section 2.4). The pH of an unknown solution will approach the conventional activity scale only if the residual liquid junction potential is small, i.e. $pH' = pH^s + \frac{(E' - E^s)}{2.303RT/F}$. This will occur only if the standard and test solutions have a similar ionic strength, solution composition, and pH. This will seldom be the case.

The problems associated with the determination of residual liquid junction potentials and single ion activity coefficients can be avoided by calibrating the electrodes against solutions of known hydrogen ion concentration with the same solution composition and ionic strength as the test solutions (Irving et al., 1967). (Alternatively, a cell without a liquid junction may be used.)

For the $p[H^+]$ range 2 - 3, the electrodes were calibrated by titration of HCl (0.0116 and 0.0232 mol L^{-1}) with KOH in 0.10 mol L^{-1} KCl. The choice of substances for hydrogen ion calibration in the $p[H^+]$ range 3.0 to 11.0 is limited. This pH range requires a buffer system. Accurate concentration quotients for the system (which have been determined at selected ionic strengths in cells without liquid junction, or with matched liquid junctions) must be available to allow the hydrogen ion concentration to be calculated. Ethylenediamine-ethylenediammoniumchloride and sodium acetate-acetic acid buffers have been used to calibrate the glass electrode as a $[H^+]$ probe in the pH range 4.0 - 10.3 (Hedwig & Powell, 1971).

In the present work, *o*-phthalic acid buffers were used to calibrate the electrodes in the $p[H^+]$ range 3.0 - 5.4 (Kennedy et al., 1983). These buffers were generated by titration of *o*-phthalic acid (4.5×10^{-3}, 4.7×10^{-3}, and 5.0×10^{-3} mol L^{-1}) with standard KOH in 0.10 mol L^{-1} KCl. Calculation of $p[H^+]$ for the buffer system at any specific composition required values of the concentration quotients for *o*-phthalic acid dissociation (Q_1 and Q_2). These quotients were calculated from the thermodynamic dissociation constants and empirical relationships for the relevant single ion activity coefficients which have been determined in KCl medium in cells without liquid junction (Hamer et al., 1945; Hamer & Acree, 1945). At $I = 0.100$ mol L^{-1}, the calculated value of log Q_1 was 2.718 and of log Q_2 was 4.945 (Kennedy et al., 1983).

A Newton-Raphson iterative procedure was used to calculate the equilibrium concentration of hydrogen ions at each datum point; initially approximate values were used for I, Q_1 and Q_2, and $10^{-pH_{NBS}}$ was used as a trial value for $[H^+]$ in subsequent iterative calculation. (Where pH_{NBS} is the measured pH corrected to the NBS scale *via* the buffer calibration data collected before and after each titration.) An approximate solution composition ($[HL^-]$ and $[L^{2-}]$) was calculated from the solution stoichiometry. This composition was then used to calculate an improved value of $[H^+]$ and I (from which improved values of Q_1 and Q_2 were obtained). A revised equilibrium solution composition was then calculated from these improved values and the iterative process was continued until the change in computed ionic strength caused by modification of $[H^+]$ was less than 0.0005 mol kg^{-1} (Kennedy et al., 1983). Only data in the well-buffered region, $p[H^+]$ range 3.0 - 5.4, were considered. A simple least squares analysis was applied to the $p[H^+]/pH_{NBS}$ data to yield a pH calibration line of the form: $p[H^+] = M\ pH_{NBS} + C$.

2.3 $p[H^+]$ Calibration Curve

A plot of measured pH against $p[H^+]$ for the titration of HCl with KOH was linear in the range $p[H^+]$ 2 - 3. However, these data were not colinear with that for the *o*-phthalic acid titrations ($p[H^+]$ 3.0 - 5.4) when extrapolated to this higher $p[H^+]$ range. For solutions of high acidity or alkalinity, the ions H_3O^+ and OH^-, which have high mobilities compared with most other ions, will make significant contributions to the liquid junction potential. Although the use of KCl as the background electrolyte minimizes liquid junction potentials due to the

approximately equal transference numbers of its ions (McBryde, 1969), the liquid junction potential for the junction between test solution and KCl (saturated) will vary with solution pH (Hedwig & Powell, 1971). At pH values greater than 3, the use of constant ionic media should reduce the liquid junction potential and its variation to negligible values (Bates, 1973). In the present work, a 'correction' was included for liquid junction effects at low pH by calculating a nonlinear regression line through the combined HCl and *o*-phthalic acid titration data to generate an equation of the form: $p[H^+] = M\ pH_{NBS} + C + D[H^+]$ (where $D[H^+]$ is the liquid junction correction term). Powell and Taylor (1983), obtained a discrepancy in pH for cells with liquid junction (compared to those without) of 0.040 at pH 1.64, 0.034 at pH 2.12, and 0.005 at pH 3.55. This would give D = -0.018 to -0.012; hence, a linear correction in $[H^+]$ may be a poor approximation.

For all $[H^+]$ calibrations, the electrodes were initially standardized against the NBS buffers as primary standards. If the same NBS buffers are used to standardize the electrodes for both the calibration titrations and the test solution measurements, then the residual liquid junction potentials for the two solutions will be identical (Hedwig, 1972). On a daily basis the electrodes need only be standardized against the NBS buffers (Kennedy et al., 1983). The electrode calibration as a $[H^+]$ probe is then valid for all subsequent measurements.

Although this approach to the calibration of glass electrodes has been criticized (May et al., 1982; May & Williams, 1985; May et al., 1985), the present author considers such a $[H^+]$ calibration to be superior to those based solely on titrations of strong acids whose anomalous liquid junction potentials make them poor standards for pH (Bates, 1973). Sjöberg, Öhman and coworkers (University of Umeä, Sweden) routinely use an acid titration as their sole means of $[H^+]$ calibration. These authors consider their method to give "a more accurate calibration than by using separate solutions of known H^+ concentration" (Marklund et al., 1986). Although this method overcomes the need for standard buffers, it involves an *in situ* calibration in each acidic test solution (at low $p[H^+]$ before ligand deprotonation or metal-ligand complexing is significant). This calibration is over only a limited $p[H^+]$ range (*ca.* 1.8 - 3.2) and the validity of a linear extrapolation of this data to higher $p[H^+]$ must be questioned.

2.4 National Bureau of Standards (NBS) Buffers

The seven primary reference pH standards established by NBS have pH values assigned to them from measurements in cells without liquid junction. The assignment involves the Bates-Guggenheim convention for γ_{Cl^-}; thus, all these standards are operationally defined. The pH scale defined by these standards is internally consistent. That is, the pH measured by a cell is approximately the same regardless of which of the seven buffers is chosen as a primary standard. The properties of these buffers have been described in several IUPAC reviews (e.g. Wu et al., 1984; Covington et al., 1985). These buffers have pH values between 3 and 11; the liquid junction potential is nearly constant over this range. Further, all these primary standards have an ionic strength less than 0.10. Deviations from this defined scale occur when pH is measured outside this range; this is due to changes in liquid junction potentials (Bates, 1973).

In the present work, three primary NBS buffers were used to standardize the electrodes both before *and* after each $[H^+]$ calibration and each titration on the test solutions. (The buffers were discarded after each measurement.) The buffers used were: 0.05 molal potassium hydrogen phthalate ($pH^s = 4.006$); 0.025 molal potassium dihydrogen phosphate/0.025 molal disodium hydrogen orthophosphate ($pH^s = 6.863$); and, 0.01 molal sodium tetraborate decahydrate ($pH^s = 9.180$) (Durst et al., 1987).

CHAPTER 3

EXPERIMENTAL

3.1 Volumetric Equipment

All glassware was 'B' grade. Pipettes and the ABU80 autoburette were calibrated by dispensing and weighing volumes of water at 25°C. The density of water at 25°C (CRC Handbook of Chemistry and Physics) was used to convert the weight of water dispensed to volume .

All glassware was cleaned by soaking in *ca.* 5% HNO_3 (BDH, Analar) for at least 48 h, followed by soaking in triply distilled water.

3.2 Titration Assembly

3.2.1 Cell Design

The double-walled titration cell was constructed from pyrex glass and had a capacity of 60 - 100 mL. The cell was fitted with an airtight lid (sealed *via* ground glass flanges) which contained ground glass 'Quickfit' entry ports for insertion of electrodes, a nitrogen bubbler, and a burette tip.

Temperature was maintained constant by circulation of thermostated water, 25 ± 0.05°C, around the jacketed cell. The titration cell was mounted on a Biolab HPS-71 stirrer; stirring of the test solution was effected *via* a Teflon-coated magnetic stirrer bar.

3.2.2 Oxygen Removal

Oxygen was removed from the test solutions by purging with oxygen-free nitrogen. Before passing through the solution, the oxygen-free nitrogen was saturated with water vapour (by bubbling the nitrogen stream through Milli-Q deionized water) at 25°C. Initial oxygen removal was effected by bubbling oxygen-free nitrogen through the solution for *ca.* 30 min; during a titration there was a continuous flow of nitrogen over the solution.

3.2.3 Automated Titration System

A Radiometer ABU80 autoburette was used to dispense standard KOH in the titration of ligand and metal-ligand solutions. The capacity of the ABU80 precision-ground glass burette (B280) was 2.5 mL; titrant was dispensed *via* a motor-driven Teflon-tipped stainless steel piston. Titrant was passed through polyethylene tubing and was dispensed into the test solution *via* a glass burette tip (D4346). The rate of addition of titrant could be adjusted from 0.125 to 4 mL min^{-1}; the slowest rate was used in the present work. The smallest volume which could be dispensed was 0.001 mL. When not in use, the titration system was filled with Milli-Q deionized water. Before each titration the "flush" program on the autoburette was activated to completely rinse the system with titrant and exclude all air bubbles (this process was performed twice prior to each titration).

The autoburette was interfaced with a Sundox microcomputer and coupled to a Radiometer PHM64 Research pH meter. The software to control the operation of the autoburette was written by Mr N. McCracken (Department of Chemistry, University of Canterbury). The program calculated the titre required to yield a particular change in pH (chosen by the user) at each datum point. At the start of a titration, the program was initiated by addition of several fixed volume increments of titre (the number and magnitude of which were chosen by the user). Following this, the program calculated the volume of titre required to give the chosen pH increment based on the change in pH resulting from addition of the previous titre volumes. The time between the addition of titre and the first pH reading at each datum point (the "delay time") was also selected by the operator. Following the first pH reading, a chosen number of pH readings were taken and the magnitude of the electrode drift was calculated. The allowable drift in pH readings was selected by the operator; the datum point was flagged if the electrode drift exceeded this limit. In the present work, a change of greater than 0.002 pH units over 30 s - 1 min was considered to be excessive drift.

Typically, the pH increment was chosen such that 40 - 80 datum points were collected for each titration.

A Sundox printer was used to record data as the titration progressed (i.e. volume of titre added, measured pH, and electrode drift). No manipulation of data was carried out by

the titration control system. The pH and volume data were then entered manually on to a VAX 6230 mainframe computer (VMS V5.3-1 operating system) for nonlinear least squares analysis (Chapter 2).

The entire titration assembly (pH meter, autoburette, titration cell, and stirrer) was placed on a 3 mm thick glass plate on top of a stainless steel bench.

3.3 Preparation of Solutions

All solutions were prepared in Milli-Q deionized water (resistance 18 MΩ). Glass spatulas were used to dispense all solid samples.

3.3.1 National Bureau of Standards (NBS) Buffers

Three primary reference NBS standard buffers were used to calibrate the glass/calomel electrode pair before and after each titration, *viz:* 0.05 molal potassium hydrogen phthalate (pH_{NBS} = 4.006), 0.025 molal di-sodium hydrogen orthophosphate/ 0.025 molal potassium di-hydrogen phosphate (pH_{NBS} = 6.863), and 0.01 molal sodium tetraborate decahydrate (pH_{NBS} = 9.180). Preparation of these buffer solutions is now described (Taylor, 1980).

Potassium Hydrogen Phthalate Buffer

Potassium hydrogen phthalate (BDH, Analar) was dried at 110°C for 1 - 2 h then stored in a desiccator over anhydrous $CaCl_2$. To prepare the buffer, 10.12 g of the dried sample was dissolved in 1 L of Milli-Q water.

Phosphate Buffer

Potassium di-hydrogen phosphate (Fisher, Analar) and di-sodium hydrogen orthophosphate (Hopkin & Williams, Analar) were dried at 40°C then stored in a dessicator over anhydrous $CaCl_2$. To prepare the buffer, 3.388 g of KH_2PO_4 and 3.533 g of Na_2HPO_4 were dissolved in 1 L of Milli-Q water.

Borax Buffer

Due to its tendency to lose water of crystallization, di-sodium tetraborate decahydrate (BDH, Analar) was not dried before use. To prepare the buffer, 3.80 g of $Na_2B_4O_7.10H_2O$ was dissolved in 1 L of Milli-Q water.

All buffer solutions were stored in a water bath at 25°C. Buffer solutions were discarded after each measurement; hence, fresh stock solutions were prepared approximately every 4 weeks.

3.3.2 Standard Alkali Solutions (KOH)

Standard KOH solutions in the concentration range 0.2 - 1.2 mol L^{-1} were prepared. Because these alkaline solutions readily absorb atmospheric CO_2 (to form insoluble carbonates which alter the hydroxide concentration), care was taken to exclude sources of carbonate contamination. Carbon dioxide-free water was prepared by boiling Milli-Q water for *ca.* 10 min followed by cooling in an ice bath under a constant stream of oxygen-free nitrogen. KOH pellets (BDH, Analar) were rinsed rapidly with the CO_2-free water to remove any carbonate coatings (the washings were discarded), then dissolved in the appropriate volume of CO_2-free water. The resulting KOH solution was standardized by titration against weighed amounts of Tris.HCl (Koch-Light, puriss). KOH solutions were stored in stoppered volumetric flasks and were restandardized every 2 - 3 weeks in case CO_2 ingress had occurred (no change in concentration over time was ever observed).

3.3.3 Standard Acid Solutions (HCl)

Two stock HCl solutions (1.124 and 1.161 mol L^{-1}) were prepared by dilution of concentrated HCl (BDH, Analar) with Milli-Q water. These solutions were standardized by titration against weighed amounts of Tris (Fluka, puriss p.a.).

3.3.4 Electrolyte Solutions (KCl)

KCl was stored in a desiccator over anhydrous $CaCl_2$ for at least 1 month before use. A stock solution of KCl, 1 mol L^{-1}, was prepared by dissolving the appropriate weight of KCl (Ajax, Analar) in Milli-Q water.

3.4 Ligand Samples and Solution Preparation

All ligands were stored in a desiccator over anhydrous $CaCl_2$. Aqueous ligand solutions were stored at 6°C. The concentrations of all ligand solutions were determined by titration with standard KOH. Ligand solutions were prepared in Milli-Q water containing the appropriate amount of KCl such that the ionic strength at the mid-point of the titration would be 0.10 mol L^{-1}.

3.4.1 Malonic Acid

Malonic acid (Riedel-de Haën, "pure") was used without further purification (unless otherwise stated). Microanalysis established the composition: C, 34.6% and H, 4.02% (c.f. calc. for $C_3H_4O_4$: C, 34.60% and H, 3.85%).

3.4.2 Isocitric Acid

DL-isocitric acid (Sigma) was supplied as the tri-sodium salt and was used without further purification. Microanalysis established the composition: C, 24.86% and H, 2.75% (c.f. calc. for $Na_3C_6H_5O_7.2H_2O$: C, 24.48% and H, 3.06%). Stock isocitrate solutions were prepared in an excess of standard HCl to generate H_3L.

3.4.3 Butane-1,2,3,4-Tetracarboxylic Acid

Butane-1,2,3,4-tetracarboxylic acid was obtained from Dr H.A. Anderson (Macaulay Institute for Soil Research, Aberdeen) and was recrystallized from 'Spectroscopic' ethanol (BDH). Microanalysis established the composition: C, 37.96% and H, 4.89% (c.f. calc. for $C_8H_{10}O_8.H_2O$: C, 38.10% and H, 4.76%).

3.4.4 Histidine

L-histidine was obtained from Sigma and was used without further purification. Microanalysis established the composition: C, 46.66%; N, 26.90% and H, 5.89% (c.f. calc. for $C_6H_9O_2N_3$: C, 46.39%; N, 27.08% and H, 5.80%).

3.4.5 5-Methoxy-N-(2-Hydroxybenzyl)Sarcosine (MHBS)

This ligand was synthesized by the method reported by Wilson (1990). Specifically, sarcosine (8.9 g, Aldrich) and paraformaldehyde (3.0 g, Ajax) were refluxed in ethanol for 1 h, resulting in formation of a pale yellow solution. *p*-methoxy phenol (12.4 g, Koch-Light) was then added and the reaction mixture was refluxed for 18 h (the resulting solution was pale orange). On cooling, white crystals of the product formed; they were collected on a porosity 4 sintered glass crucible and washed with a small volume of cold ethanol. The product was recrytallized from 45% ethanol/triply distilled water.

The melting point of the ligand was 199 - 201°C c.f. 196 - 198°C reported by Wilson (1990). Microanalysis of the synthesized ligand established the composition: C, 58.68%; N, 6.47% and H, 6.48% (c.f. calc. for $C_{11}H_{14}O_4N$: C, 58.93%; N, 6.25% and H, 6.25%).

3.4.6 *o*-Phthalic Acid

The *o*-phthalic acid sample was synthesized by Kennedy et al. (1983).

3.5 Metal Solutions

3.5.1 Copper(II)

$Cu(NO_3)_2.3H_2O$ (BDH, Analar) was dissolved in 8.96×10^{-4} mol L^{-1} HCl. The concentration of this solution was determined gravimetrically as the benzoin-α-oximate and electrolytically by deposition on a platinum cathode; both these procedures are described by Vogel (1974). The concentration of this Cu(II) solution was 0.1036 mol L^{-1}.

3.5.2 Aluminium(III)

Two stock Al(III) solutions were prepared by dissolving $AlCl_3.6H_2O$ (Riedel-de Haën, Chem. pure) in *ca.* 0.10 mol L^{-1} HCl. The Al(III) content of these solutions was determined gravimetrically (in triplicate) as the oxinate (Vogel, 1974). These solutions were 0.1227 and 0.1516 mol L^{-1} in Al(III). A purer Al(III) solution was prepared from ALFA $AlCl_3.6H_2O$ (99.9995%). This solution was 0.1616 mol L^{-1} in Al(III).

The acid content of each solution was determined by titration against standard KOH. These solutions were stored in acid-washed polyethylene containers at 6°C.

3.6 Metal-Ligand Solutions

For potentiometric studies, metal-ligand solutions (Al(III)-malonate, -isocitrate, and Cu(II)-tetrabutanoate, -MHBS) were prepared by mixing the appropriate volumes of the standardized stock metal and ligand solutions, HCl and KCl (to give an ionic strength of 0.10 mol L^{-1} at the mid-point of the titration). The stock solutions were equilibrated at 25°C before volumes were dispensed; the metal-ligand solution was equilibrated at 25°C before being made up exactly to the final volume. The metal-ligand solutions were stored at 6°C.

3.7 Titration Procedure

The procedure followed for potentiometric titrations of ligand, or of metal-ligand solutions is now described.

Buffers and the test solutions were purged with oxygen-free nitrogen; measurements on these solutions were performed in the same titration cell. Before every titration, the glass/calomel electrode pair was standardized against the three NBS buffers; *ca.* 30 min was allowed for a stable pH reading to be obtained. The solution to be titrated was equilibrated at 25°C then the appropriate aliquot was pipetted into the titration cell; *ca.* 30 min was allowed for a stable pH reading to be obtained before the titration was commenced. Titrant was then dispensed *via* the automated titration system described in Section 3.2.3. At the completion of every titration the electrodes were re-standardized against the same three NBS buffers to allow correction to be made for any drift in

the electrode response over time (which was assumed to have been linear over the course of the titration).

3.8 Electrodes

Several glass electrodes were used, *viz:* 4 were Beckman electrodes with a type E-2 glass membrane (low sodium ion error), one was a Radiometer (G202B), and one was a Russell electrode (SWR757). The calomel electrodes used were from Beckman (39417) and Russell (CR5) with saturated KCl (Analar) as the electrolyte solution.

The glass/calomel electrode pairs were calibrated as described in Chapter 2. The pH measurements were made with a Radiometer PHM64 Research pH meter. When not in use, the glass and calomel electrodes were stored in 0.10 mol L^{-1} KCl at 25°C.

3.9 Humic Substances

All solid humic samples were stored in a desiccator over anhydrous $CaCl_2$. Aqueous humic solutions were kept in the dark at room temperature for short term storage; solutions used less frequently were stored at 6°C.

3.9.1 Fulvic Acids

Two fulvic acid samples were used in the present work. Both samples were extracted by Gregor (1987) using the acid-pyrophosphate - XAD-7 method (Gregor & Powell, 1986a). FA2 was extracted from a Maimai gley podzol, West Coast, South Island, New Zealand (B_h subhorizon); FA4 was extracted from an International Humic Substances Society (IHSS) bog peat; FAS was an alkali extracted sample obtained from Dr M. Schnitzer (Chemistry and Biology Research Institute, Agriculture Canada, Ontario).

The composition of these samples was (Cressey et al., 1983; Gregor, Powell and Town, 1989a):

FA2: C, 50.1%; H, 3.8%; N, 0.6%; P, 0.10%; Si, 0.09%; Al, 0.07%; Fe, 0.06%.

FA4: C, 49.3%; H, 3.7%; N, 2.3%; P, 0.06%; Si, 0.06%; Al, 0.01%; Fe, 0.05%.

FAS: C, 43.8%; H, 3.6%; N, 0.6%; P, 0.17%; Si, 0.65%; Al, 0.02%; Fe, 0.08%.

The equivalent weights were: FA2, 142; FA4, 139; FAS, not determined (Gregor, 1987). The ash contents were: FA2, 0.6%; FA4, 0.2%; FAS, 1.2%.

Analysis by ICP-MS established trace impurities (ppm) for FA4 of Cu (61), Pb (<38), Cd (<3.8), Zn (23), Al (107), and Fe (575); for FA2 of Cu (27.6), Pb (<39), Cd (<3.9), Zn (31), Al (169), and Fe (224); and for FAS of Cu (249), Pb (<38), Cd (<3.8), Zn (23), Al (153) and Fe (238).

Fulvic acid solutions were prepared by dissolving the appropriate weight in Milli-Q water.

3.9.2 Humic Acid

The humic acid was an IHSS reference soil humic acid (Summit Hill), SHHA. Elemental analysis (provided by Prof. R.S. Swift, University of Reading) established: C, 49.5%; H, 5.01%; N, 4.67% and S, 0.59% (these data were supplied as mean values on an ash-free basis). The ash content was 1.15%. Analysis by ICP-MS established trace impurities (ppm) of Cu (52.3), Pb (<65), Cd (<6.5), Zn (<6.5), Al (209), and Fe (203). Neutron activation analysis (performed by Dr J.J. Fardy, ANSTO, Lucas Heights, Sydney) established trace impurities (ppm) of Na (309), Cl (980), K (2 490), and Br (206). The X-ray powder diffractogram was measured for SHHA in the 2 Θ range 7 - 30 (Philips PW 1729 x-ray generator and PW 1710 diffractometer control). This established that there was no detectable crystalline material present in the sample.

Preparation of Humic Acid Solutions

Humic acid is, by definition, insoluble in acidic solutions. Therefore, to effect dissolution and to obtain a 'representative' humic acid solution (Chapter 5), the SHHA was initially 'pre-dissolved' in *ca.* 0.8 mol L^{-1} KOH, stood for *ca.* 1 h, then diluted to the appropriate volume and the pH adjusted to *ca.* 7 by addition of Aristar HNO_3. Stock SHHA solutions of *ca.* 1 mg mL^{-1} were prepared in this manner. For filtered SHHA solutions, membrane filtration (0.025 μm) was effected on the pH 7 solution (unless otherwise stated). The concentration of the filtered SHHA solution was determined by UV-visible

spectroscopy; an unfiltered solution of known concentration and the filtered solution were both diluted in *ca.* 0.8 mol L^{-1} KOH (to 'completely' dissolve the SHHA), then their UV-visible absorption spectra were recorded. The concentration of the filtered SHHA solution could then be calculated by comparison of its absorbance at several wavelengths with that for the unfiltered solution. Typically, the concentration of a filtered SHHA solution was *ca.* 25% less than that of the corresponding unfiltered solution.

Determination of the Equivalent Weight of SHHA

The equivalent weight (weight/mole of titratable carboxyl groups) was determined by titration with standard KOH. An end point inflexion was observed in the pH range 5 - 9. Data beyond the end point (within one pH unit) were used in the Gran's analysis to determine the end point titre volume. Data were used in a limited pH range only, due to the heterogeneity of humic acid and the likely deprotonation of other functional groups beyond this range.

Two titrations were performed on separate weighed samples of SHHA in 0.1 mol L^{-1} KCl; one 'fast' (a 2 min delay time between the addition of KOH and the first pH reading), the other 'slow' (a 1 h delay time). The equivalent weight determined by the slow titration was 287; that determined by the fast titration was *ca.* 3% greater than this. This result indicates that proton exchange on humic acid is very fast, even in the presence of particulate matter (solid material was visible in solution throughout the titration).

The equivalent weight of humic acid samples determined by other workers includes: 144 for a peat humic acid (Pommer & Breger, 1960a); 150 for Merck 'humic' acid (Pommer & Breger, 1960b); and, 345 for Fluka 'humic' acid (van den Hoop et al., 1990);

3.9.3 Filtration Procedure

Humic samples were filtered through 0.025 μm membranes (Schleicher & Schuell, 47 mm and 25 mm diameter). The larger diameter filters were used in conjunction with a Satorius SM 16510 filtration unit (capacity, 250 mL); suction was applied *via* a water pump to effect filtration. For small sample volumes, the 25 mm diameter filters were used in

conjunction with a hand held filtration unit which allowed the samples to be passed through the filter *via* a 5 mL disposable syringe. In all cases the filtration apparatus (which did not contain any metallic parts) was acid washed before use. Each membrane filter was rinsed with a small volume of dilute Aristar HNO_3, followed by Milli-Q water, before filtration of the humic sample.

3.10 Analytical Procedures

3.10.1 Microanalysis

Elemental analysis of samples (C, H, and N) was performed at the University of Otago. Humic samples were dried to constant weight at 70°C prior to analysis; all other samples were stored over silica gel for 48 h.

3.10.2 Inductively Coupled Plasma - Mass Spectrometry (ICP-MS)

ICP-MS was used to determine the inorganic trace impurities in the humic samples. Analyses were performed by Mr L. Dale, CSIRO, Lucas Heights, Sydney. Fulvic acids were dissolved in water (*ca.* 25 mg/10 mL); SHHA was prepared in dilute HNO_3 (15 mg/10mL).

3.10.3 Flame Atomic Absorption Spectroscopy (FAAS)

FAAS (Varian AA-1475) was used to determine the potassium, calcium and sodium content of a malonic acid solution (8.3×10^{-3} mol L^{-1}). Sodium and potassium were measured by flame emission at 588 nm and 765 nm respectively; calcium was measured by flame absorption at 421 nm.

3.10.4 Nuclear Magnetic Resonance (NMR)

^{1}H and ^{13}C NMR were recorded for aqueous solutions on a Varian XL300 spectrometer.

CHAPTER 4

pH POTENTIOMETRIC STUDIES ON THE COMPOSITION AND STABILITY OF Al(III) AND Cu(II) COMPLEXES WITH CARBOXYLATE LIGANDS

4.1 INTRODUCTION

This chapter describes quantitative solution equilibria studies on the complexation of Al(III) and Cu(II) by carboxylate ligands. Experimental details are given in Chapter 3 and the methodology for the calculation of stability constants from pH potentiometric titration data is described in Chapter 2.

As noted in Chapter 1, the heterogeneous and ill-defined nature of humic substances prevents quantitative analysis of their complexation with metal ions. Hence, studies on the complexation of metal ions by simple carboxylate ligands which are important environmentally, and which may be suitable models for the chelating moieties in humic substances, was considered to be a reasonable approach to this problem. Specifically, the ligands studied were malonic acid, isocitric acid, butane-1,2,3,4-tetracarboxylic acid (tetrabutanoic acid), and 5-methoxy-N-(2-hydroxybenzyl)sarcosine (MHBS). The metal ions studied were Al(III) and Cu(II).

Aluminium is the most abundant metal in the earth's crust and is an important metal ion in soil solutions. Its oxides and silicates are highly insoluble; thus, it is generally unavailable to participate in biogeochemical reactions (Driscoll & Schecher, 1990). However, the increased mobilization of Al(III) into ground waters, and the concomitant increase in toxicity to aquatic organisms, as a result of acid precipitation has generated interest in the chelation and speciation of Al(III) in the environment.

Humic substances are the predominant organic ligands in soils and natural waters and as such they may be important in controlling the speciation of Al(III) in the environment. Indeed, humic-bound Al(III) is considered to be nontoxic to plants and microorganisms (Gunn et al., 1986; Stevenson & Vance, 1989). Aluminium complexation occurs predominantly with oxygen-containing functional groups; ligands containing nitrogen donors

generally form weak complexes with Al(III). Hence, the ligands which will be important in governing Al(III) speciation are those containing carboxyl (aliphatic or aromatic) and hydroxyl groups (either phenolic, enolic, or aliphatic).

In the present work, Al(III) complexation with the ligands malonic acid and isocitric acid was studied. These ligands were considered to be appropriate models for the functional groups in humic substances. According to Pott et al. (1985), the stability constants for the complexation of Al(III) by humic acid are similar to those for citric acid. Coupled with the pH dependence of humic acid-Al(III) binding capacities these results were proposed to be consistent with a carboxylate mode of coordination for humic substances with aluminium.

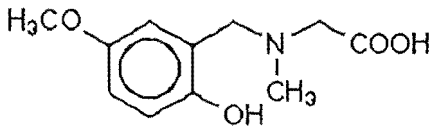
Malonic acid was chosen because computer modelling studies of the pH dependent complexation of Cu(II) by fulvic acid indicated that it may be an important chelating group in humic substances (Gregor, Powell & Town, 1989a,b). A recent study by Leenheer et al. (in preparation) proposed that the acidity characteristics of Suwannee River fulvic acid were consistent with the presence of a significant quantity of substituted malonic acid functional groups. The Al(III)-malonate system has been studied previously; however, the values reported for the stability constants are not in good agreement.

Modelling of Cu(II)-fulvic binding also indicated that citrate may be an important moiety in humic substances. The Al(III)-citrate system has been studied extensively; however, conflicting models have been reported (e.g. Gregor & Powell, 1986b; Öhman & Sjöberg, 1983; Öhman, 1988). To the author's knowledge, stability constants for the complexation of Al(III) by isocitrate have not been reported. A study of the Al(III)-isocitrate system was intended to further elucidate the coordination of citrate to Al(III) which produces an exceptionally stable complex.

Some of the variation in models reported for complexation of Al(III) by simple ligands could be due to the very slow kinetics of Al(III) complexation (Öhman, 1988). In addition, Al(III) is a readily hydrolyzable metal ion. The hydrolysis has been studied extensively, with conflicting results being reported (Bottero et al., 1980; Brown et al., 1985; Letterman & Asolekar, 1990a,b). It is very difficult to establish the composition and stability of hydrolytic Al(III) species due to the formation of polynuclears, the extremely slow attainment of

equilibria, and the interference from transient and permanent precipitates (Hedlund et al., 1987).

Copper(II) is also an environmentally significant metal ion which forms very stable complexes with humic substances. Cu(II) complexation occurs predominantly with ligands containing oxygen and nitrogen or sulphur donor groups. Ion selective electrode potentiometric studies have indicated that neither the Cu(II) binding strength nor the Cu(II) binding capacity of fulvic acid can be adequately described by any one simple model ligand (Gregor, Powell & Town, 1989a,b). To provide better agreement with the Cu(II)-fulvic acid binding curves, additional ligand moieties which bind Cu(II) strongly and which deprotonate at pH >5.5 needed to be included in the model. Nitrogen containing ligands may contribute to strong Cu(II) binding by humic substances. However, the nitrogen content of humic substances is low (in the range 0.5 - 5%); further, it has not been completely characterized. Another possibility which may account for this 'shortfall' in Cu(II) binding is the formation of more stable intermolecular ternary complexes. A related concept is enhanced intramolecular coordination of weak donor groups (e.g. phenolic hydroxyl) by virtue of their proximity to primary coordination sites such as malonate, citrate, or peptide moieties (cascade binding). To investigate systems of this type, complexing between Cu(II) and MHBS (shown below) was studied.



5-methoxy-N-(2-hydroxybenzyl)sarcosine (MHBS); H_2L

The formation of Cu(II) complexes with tetrabutanoic acid was also investigated. Such groupings of carboxyl groups are likely to occur in humic substances (Bracewell et al., 1980).

4.2 RESULTS

Stability constants were determined by titration of standard KOH into ligand, or metal-ligand, solutions at constant ionic strength (0.10 mol L^{-1} KCl) and temperature (25°C).

In the titration of all ligands with standard KOH, the concentration of ligand obtained from the end point of the titration (*via* Gran's analysis of data beyond the end point) was less than that expected from the weight of material used. This discrepancy, typically 1 - 2%, was ascribed to a small amount of moisture associated with the solid samples. The total acid for each titration (TH) was fixed at the Gran's-determined value (unless otherwise stated). In the metal-ligand titrations, the concentration of ligand was obtained from a separate titration on the stock ligand solution. Standardization of the ligand concentration is critical in these measurements; for example, a small error in the concentration of a tetraprotic ligand would produce a corresponding 4-fold error in the concentration of titratable hydrogen ions.

Two types of calculation were performed: refining on TH (which allowed TL to be a variable parameter), and refining on ñ. Lower R-factors were obtained for refinements on the basis of TH due, in part, to the presence of an additional variable parameter (May et al., 1988). In general, when the concentration of ligand was allowed to vary in the least squares calculations, the value obtained for this parameter was very close to TH/n for a ligand with n titratable protons. For calculations which refined on ñ, the value of TL was fixed at the least squares refined value obtained from the calculations which refined on TH.

For the calculation of metal-ligand stability constants, the values of the ligand protonation constants used in the nonlinear least squares refinement process included K^+ ion pairing (if this data was available) and correction for ionic strength variations over the course of the titration.

4.2.1 Calibration of the Glass Electrode as a Hydrogen Ion Concentration Probe

To calibrate the glass electrode as a hydrogen ion concentration probe (as described in Chapter 2, Section B), *o*-phthalic acid and standard HCl solutions were titrated with standard KOH.

Four titrations were performed on *o*-phthalic acid solutions (4.86×10^{-3} mol L^{-1}, 4.64×10^{-3} mol L^{-1} (2 titrations), and 4.66×10^{-3} mol L^{-1}). Only data in the well buffered region, $p[H^+]$ 3.0 - 5.4, were considered with an average of 30 datum points per titration.

Five titrations were performed on standard HCl solutions (0.0232 mol L^{-1} (3 titrations) and 0.0116 mol L^{-1}). Data in the $p[H^+]$ range 2.0 - 3.0 were considered (where the plot of $p[H^+]$ *versus* pH_{meas} was linear), with an average of 10 datum points per titration.

The data for these nine *o*-phthalic acid and HCl titrations were pooled and a nonlinear regression line was calculated through all datum points (188 in total). The line of best fit was: $p[H^+] = 1.000\ pH_{NBS} - 0.08662 + 3.832\ [H^+]$.

4.2.2 Testing of the Apparatus and Verification of the Calculation Procedure used for the Determination of Stability Constants

Stability constants for the reaction of Cu(II) with histidine have been measured by many workers and these data have been critically surveyed by IUPAC (Pettit, 1984). Solutions of Cu(II)-histidine at 1:1 (2.59×10^{-3} mol L^{-1} Cu(II), 2.99×10^{-3} mol L^{-1} histidine) and 1:2 (2.07×10^{-3} mol L^{-1} Cu(II), 4.78×10^{-3} mol L^{-1} histidine) metal-to-ligand ratios were titrated (in 0.10 mol L^{-1} KCL at 25°C). Five titrations were performed with an average of 29 datum points per titration (145 in total). The IUPAC recommended protonation constants for histidine were used in the calculations, *viz:* $\log K_{HL} = 9.11$, $\log K_{H_2L} = 6.05$, $\log K_{H_3L} = 1.72$ (Pettit, 1984). Titration data in the $p[H^+]$ range 3.0 - 5.8 were used to calculate the Cu(II)-histidine stability constants using the ORGLS computer program. IUPAC recommended values for the stability constants are compared with those calculated in the present work in Table 4.1.

Table 4.1: Stability Constants for Cu(II) - L-Histidine Complexation

Complex	log β (R)[a]	log β[b]
CuHL	14.11 (0.02)	14.18 (0.09)
CuL	10.16 (0.03)	10.20 (0.06)
CuL_2	18.11 (0.09)	18.2 (0.3)
CuLOH	2.0 (0.2)	2.26 (0.03)
$CuHL_2$	23.81 (0.07)	23.83 (0.08)
CuH_2L_2	27.2 (0.1)	c

Errors are given in parentheses.

[a]IUPAC recommended values.

[b]determined in the present work; error is ± 3σ.

[c]this value was fixed at the recommended value in the least squares calculations.

The effect of solution composition errors on the calculated stability constants was assessed. A 0.3% error in the concentration of KOH, histidine, or Cu(II) changed the calculated β values by a maximum of 2%.

4.2.3 Al(III) - Malonic Acid Equilibria

Malonic Acid Protonation Constants

A total of 8 titrations at 3 ligand concentrations (3.5×10^{-3}, 4.5×10^{-3} and 9.5×10^{-3} mol L^{-1}) were performed. Data in the $p[H^+]$ range 2.8 - 5.8 were used to calculate the protonation constants, with an average of 65 datum points per titration (523 in total). The titration curve ($p[H^+]$ *versus* volume of KOH titre) exhibited no inflexions until the end point (which corresponded to titration of 2.0 protons per ligand); the buffer region was $p[H^+]$ 3.5 - 5.0). Values calculated for log K_1 and log K_2 are given in Table 4.2. Small changes in ionic strength occurred during these titrations; the effect of this on the calculated protonation

constants was assessed (see Chapter 2) but was found to be insignificant for these concentrations of diprotic ligand. The results for the inclusion of ion-pair formation, K^+L^{2-} (log K = 0.68; Daniele et al., 1985b) are also given in Table 4.2.

Calculations performed by refining on TH or on ñ resulted in the same protonation constants.

Table 4.2: Protonation Constants for Malonic Acid: 25°C, I = 0.1 M KCl

Reaction	Constant	a	b
$L^{2-} + H^+ \leftrightharpoons HL^-$	$\log K_1$	5.25 ± 0.05	5.43 ± 0.08
$HL^- + H^+ \leftrightharpoons H_2L$	$\log K_2$	2.60 ± 0.08	2.60 ± 0.08

Data are reported as mean ± 3σ for 8 titrations.

[a]Assuming fixed ionic strength, I = 0.1 mol L^{-1} KCl.

[b]K^+L^{2-} ion pairing included.

When the concentration of ligand (TL) was allowed to vary in the least squares calculations the value obtained was approximately equal to TH/2 for titrations on samples of malonic acid which had not been recrystallized. For recrystallized malonic acid, the least squares value of TL was 1 - 2% *greater* than TH/2. This result is discussed in Section 4.3.3. For the protonation constants reported in Table 4.2, TL was fixed at TH/2.

Al(III)-Malonic Acid Stability Constants

The stability constants for Al(III) complexation by malonic acid were determined from 4 titrations at metal:ligand ratios of 1:2 (1), 1:3 (2), and 1:5 (1). The concentrations of metal and ligand used in these titrations were, respectively: 2.424 x 10^{-3} mol L^{-1} Al(III), 5.100 x 10^{-3} mol L^{-1} malonic acid; 1.616 x 10^{-3} mol L^{-1} Al(III), 4.896 x 10^{-3} mol L^{-1} malonic acid; and 9.696 x 10^{-4} mol L^{-1} Al(III), 4.896 x 10^{-3} mol L^{-1} malonic acid. Data in the $p[H^+]$ range 2.20 - 5.50 were used in the nonlinear least squares calculations. All data used unrecrystallized

malonic acid (see Section 4.3.3). There was no inflexion in the titration graph until the end point (which corresponded to titration of 2.0 protons per ligand plus added acid); there was a buffer region in the $p[H^+]$ range 4.0 - 5.5.

For titrations at 1:3 and 1:5 Al:malonic acid ratios, ZC did not exceed 2.0 below $p[H^+]$ 6.4. At a 1:2 ratio ZC became greater than 2.0 above $p[H^+]$ 5.7, indicating formation of hydroxy species. There was a minor inflexion in the Bjerrum plots ($\bar{n}$ *versus* pL) at $\bar{n} = 2$, indicating the formation of AlL_2; $\bar{n}$ values greater than 2.0 indicated that some AlL_3 was also formed (Figure 4.1). These $\bar{n}$/pL plots were coincident for the different absolute concentrations of Al(III) and malonic acid and the different metal:ligand ratios in the $p[H^+]$ range 2.2 to *ca.* 5.0. Above $p[H^+]$ 5.0 some deviation from a coincident family of plots was observed, indicating that hydroxy species could be contributing to the solution composition. The Al(III) hydroxy species $Al(OH)^{2+}$ (log β = -5.52), $Al_3(OH)_4^{5+}$ (log β = -13.57), and $Al_{13}O_4(OH)_{24}^{7+}$ (log β = -109.2) were included in the equilibrium model (Öhman & Forsling, 1981; Hedlund et al., 1987).

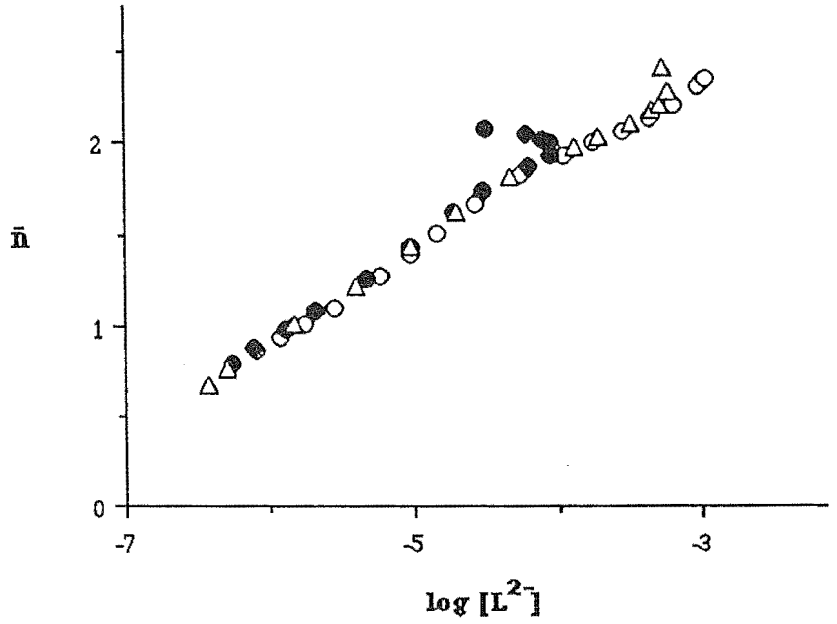


Figure 4.1: Bjerrum plots for the Al(III)-Malonic Acid System

O 1:5 Al(III):malonic acid ratio, Δ 1:3, ● 1:2

The stability constants for the complexation of Al(III) by malonic acid are given in Table 4.3; the pH dependent species distribution for titration data at a 1:3 ratio is given in Figure 4.2.

Table 4.3: Stability Constants for Al(III)-Malonic Acid Complexation: 25°C, I = 0.1 M KCl

Reaction	log K
$Al^{3+} + L^{2-} \leftrightharpoons AlL^{+}$	6.71 ± 0.03
$AlL^{+} + L^{2-} \leftrightharpoons AlL_2^{-}$	4.94 ± 0.02
$AlL_2^{-} + L^{2-} \leftrightharpoons AlL_3^{3-}$	2.61 ± 0.04
$AlL_2^{-} \leftrightharpoons AlL_2OH + H^{+}$	-7.0 ± 0.1

Data are reported as mean ± 3σ for 4 titrations.

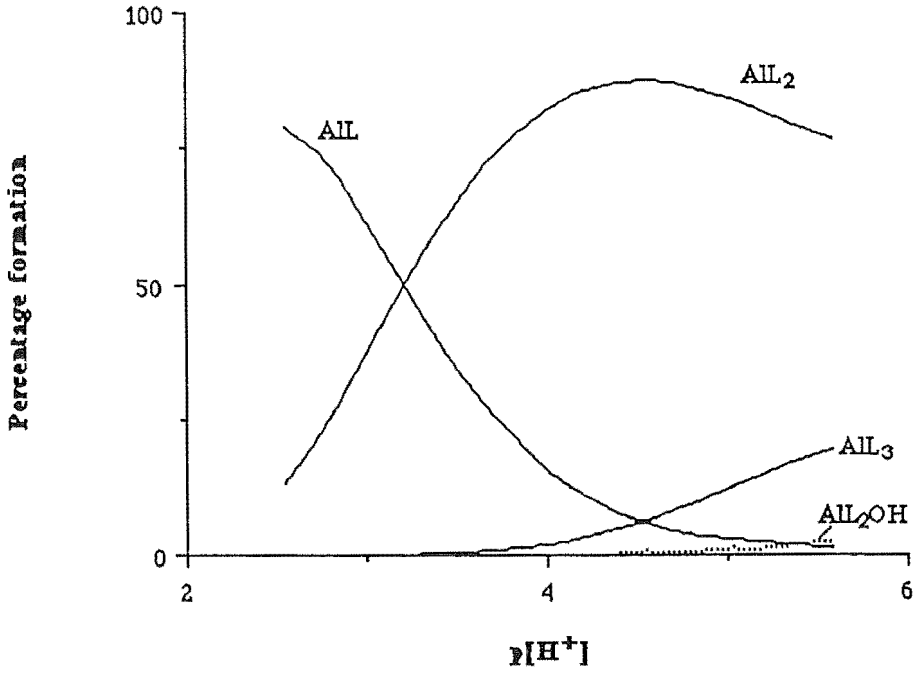


Figure 4.2: Species Distribution for the Al(III)-Malonic Acid System

4.2.4 Al(III)-Isocitric Acid Equilibria

Isocitric Acid Protonation Constants

Five titrations at 2 ligand concentrations were performed (4.5×10^{-3} mol L^{-1} and 3.5×10^{-3} mol L^{-1}). Values for log K_n (n = 1 - 3) are given Table 4.4. Data in the p[H^+] range 2.9 - 6.0 were used in the calculations; there were approximately 60 datum points in each titration (298 in total). There was no inflexion in the titration graph until the end point (which corresponded to titration of acid added to the salt Na_3isocitrate). Calculations performed by refining on ñ or on TH resulted in the same protonation constants. The effect of including changes in ionic strength throughout the titration, and consideration of K^+L^{3-} ion pairing (log K = 0.77 (Rechnitz & Zamochnick, 1964)), are also reported.

Table 4.4: Protonation Constants for Isocitric Acid: 25°C, I = 0.1 M KCl

Reaction	Constant	a	b	c	d
$H^+ + L^{3-} \leftrightharpoons HL^{2-}$	log K_1	5.64 ± 0.05	5.68 ± 0.04	5.88 ± 0.04	5.88 ± 0.04
$H^+ + HL^{2-} \leftrightharpoons H_2L^-$	log K_2	4.28 ± 0.04	4.30 ± 0.04	4.30 ± 0.04	4.29 ± 0.05
$H_2L + H^+ \leftrightharpoons H_3L$	log K_3	3.09 ± 0.05	3.10 ± 0.05	3.10 ± 0.05	3.09 ± 0.05

Data are reported as mean ± 3σ for 5 titrations.

[a]Including variation in ionic strength; refining on TH.

[b]Assuming fixed ionic strength; I = 0.1 mol L^{-1} KCl; refining on ñ.

[c]Assuming fixed ionic strength; K^+L^{3-} ion pairing considered; refining on ñ.

[d]Including correction for variation in ionic strength (ion pairing included); refining on ñ.

Because this ligand was supplied as the sodium salt ($Na_3L.2H_2O$), excess acid (HCl) was added to generate H_3L before titration of the ligand solution with KOH. The presence of this excess acid meant that the value of TL could not be obtained directly from TH for the titration. Therefore, the initial data analysis was performed by refining on TH with

TL as a variable parameter. This least squares refined value of TL was then used in the calculations which refined on ñ.

Al(III)-Isocitric Acid Stability Constants

The Russell electrodes and the high purity Al(III) solution were used for these measurements (Section 4.3.4).

The time required for the Al(III)-isocitrate system to reach equilibrium was measured. The glass/calomel electrode pair was equilibrated in a freshly prepared acidic Al(III)-isocitric acid solution (5 x 10^{-3} mol L^{-1} in both Al(III) and isocitrate), an aliquot of KOH was then added quickly with rapid stirring (to generate $p[H^+]$ values of *ca.* 4.0, 5.5, and 7.5). The resulting $p[H^+]$ values were monitored over time until equilibrium was attained (i.e. pH values remaining constant to 0.002 pH over 1 h). At $p[H^+]$ 4.0 and 5.5, $p[H^+]$ readings drifted to more acidic values; equilibrium was attained within 1 h. The change in $p[H^+]$ observed over this time was 0.03 at $p[H^+]$ 4.0, and 0.15 at $p[H^+]$ 5.5. At $p[H^+]$ 7.5 the initial drift was to more alkaline values (by 0.04); after 1 h the $p[H^+]$ values slowly became more acidic, attaining a stable value after 20 h (the final $p[H^+]$ was 0.07 more acidic than that measured 5 min after the addition of KOH).

The rate of pH drift observed for the Al(III)-isocitric acid system below $p[H^+]$ 6 is much less than that reported for Al(III)-citric acid solutions (Öhman, 1988). This reasonably rapid attainment of equilibrium allowed the stability constants for this system to be calculated from direct titration of Al(III)-isocitric acid solutions. Five titrations were performed at metal-to-ligand ratios of 1:1.9 (1), 1:3 (2), and 1:5 (2). The concentrations of metal and ligand used in these titrations were, respectively: 1.895 x 10^{-3} mol L^{-1} Al(III), 3.600 x 10^{-3} mol L^{-1} isocitric acid; 1.263 x 10^{-3} mol L^{-1} Al(III), 3.837 x 10^{-3} mol L^{-1} isocitric acid; and, 6.464 x 10^{-4} mol L^{-1} Al(III), 3.426 x 10^{-3} mol L^{-1} isocitric acid.

There was no inflexion in the titration curve until the end point which corresponded to titration of 3.0 protons per ligand plus $1\frac{1}{3}$ protons per Al(III) (consistent with the end point stoichiometry reported for Al(III)-citrate (Öhman, 1988)). The titration curves for a 1:1.9

Al(III):isocitric acid solution, and that for the same concentration of isocitric acid alone are given in Figure 4.3.

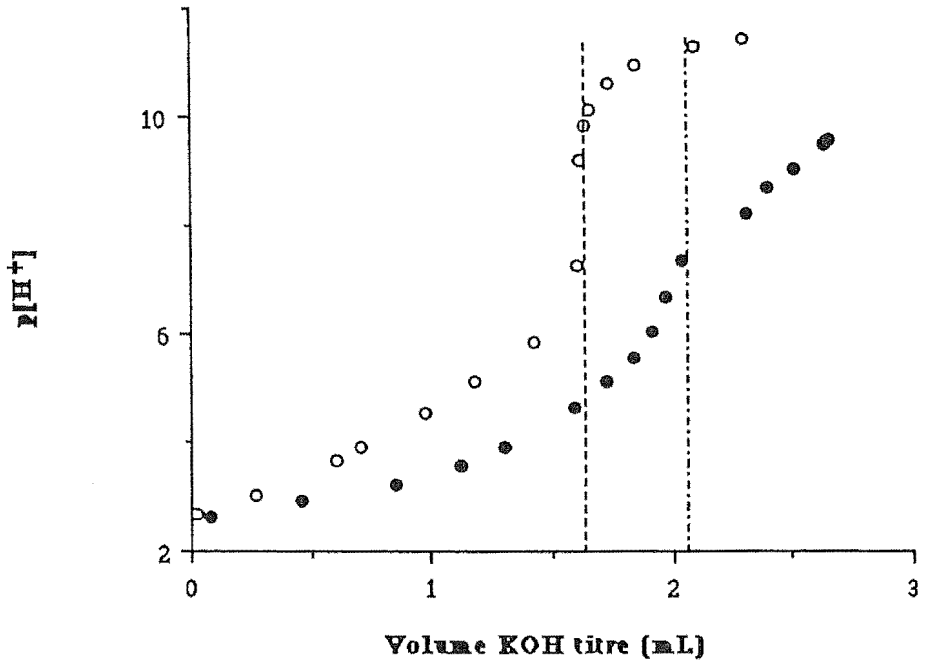


Figure 4.3: Isocitric Acid (o) and Al(III)-Isocitric Acid (•) Titration Curves

[isocitric acid] = 3.600 x 10^{-3} M, [Al(III)] = 1.895 x 10^{-3} M.

- - - Titre volume corresponding to titration of 3.0 protons per ligand;

- · - · Titre volume for titration of 3.0 protons per ligand + $1\frac{1}{3}$ protons per Al(III).

Data in the p[H+] range 2.3 - 4.0 were described by the formation of $AlHL^+$ (log β = 10.07 ± 0.03) and AlL (6.96 ± 0.08) (constants are reported as the mean ± 3σ for 5 titrations). Extensive analysis of the data beyond this p[H+] range was not attempted. The Al(III) hydroxy species given in Section 4.2.3 were included in the equilibrium model.

4.2.5 Cu(II)-Tetrabutanoic Acid Equilibria

Tetrabutanoic Acid Protonation Constants

To determine the protonation constants for tetrabutanoic acid a total of 6 titrations at 3 ligand concentrations (3.95×10^{-3}, 5.2×10^{-3}, and 7.5×10^{-3} mol L^{-1}) were performed. There was no inflexion in the titration graph until the end point (corresponding to titration of 4.0 protons per ligand). Data in the $p[H^+]$ range 2.9 - 6.5 were used in the calculations with an average of 78 datum points per titration (465 in total).

Values for log K_n (n = 1 - 4) are given in Table 4.5. This table also includes results for consideration of ionic strength variation throughout the titration. To the authors knowledge, no data is available for K^+ ion pairing with tetrabutanoic acid; hence, this factor could not be considered in the calculations. Calculations were performed by refining on TH and by refining on ñ; there was no significant difference in the mean protonation constants calculated by these 2 methods (Table 4.5).

Table 4.5: Protonation Constants for Tetrabutanoic Acid: 25°C, I = 0.1 M KCl

Reaction	Constant	a	b	c	d
$H^+ + L^{4-} \leftrightharpoons HL^{3-}$	log K_1	6.27 ± 0.05	6.22 ± 0.04	6.27 ± 0.05	7.16
$H^+ + HL^{3-} \leftrightharpoons H_2L^{2-}$	log K_2	5.18 ± 0.05	5.19 ± 0.05	5.18 ± 0.05	5.85
$H^+ + H_2L^{2-} \leftrightharpoons H_3L^-$	log K_3	4.12 ± 0.06	4.14 ± 0.03	4.12 ± 0.03	4.56
$H^+ + H_3L^- \leftrightharpoons H_4L$	log K_4	3.19 ± 0.08	3.19 ± 0.07	3.17 ± 0.08	3.43

Data are reported as mean ± 3σ for 6 titrations.

[a]Including correction for variation in ionic strength; refining on TH.

[b]Assuming fixed ionic strength (I = 0.1 M KCl); refining on ñ.

[c]Including correction for variation in ionic strength; refining on ñ.

[d]Data from Purdie et al. (1972), 25°C.

Cu(II)-Tetrabutanoic Acid Stability Constants

Precipitation occurred when Cu(II)-tetrabutanoic acid solutions were titrated. In a solution containing 4.9 x 10^{-3} mol L^{-1} tetrabutanoic acid and 2.1 x 10^{-3} mol L^{-1} Cu(II) (in 0.1 mol L^{-1} KCl), a pale blue precipitate was observed at p[H^+] *ca.* 3.5. A range of metal:ligand ratios and concentrations was investigated to see if there were any experimental conditions under which precipitation could be prevented. However, precipitation occurred even with 5 x 10^{-4} mol L^{-1} Cu(II) and 1 x 10^{-3} mol L^{-1} tetrabutanoic acid (the minimum concentrations required for the potentiometric measurements).

4.2.6 Cu(II) - 5-Methoxy-N-(2-Hydroxybenzyl)Sarcosine (MHBS) Equilibria

MHBS Protonation Constants

The protonation constants for this ligand are sufficiently separated to allow each constant to be determined individually. There were inflexions in the titration curve corresponding to titration of 1.0 and 2.0 protons per ligand plus added acid; there was no inflexion for titration of the 3rd ligand proton. Protonation constants for this ligand were calculated by refining on TH. If the concentration of ligand was allowed to vary, the value obtained was the same as the concentration expected on the basis of the weight of ligand used to prepare the solutions.

The value of log K_3 indicates a high acidity. Therefore, in order to determine this value as reliably as possible, the electrodes were calibrated by an *in situ* HCl-KOH titration prior to the addition (and subsequent titration) of the ligand. Values of p[H^+]*versus* pH_{meas} for this titration were used to convert the measured pH values for the ensuing ligand titration to p[H^+] values in the nonlinear least squares calculations. A total of 4 titrations at 4.70 x 10^{-3} mol L^{-1} ligand were performed; the average number of datum points per titration was 23 (92 in total). Data in the p[H^+] range 2.3 - 4.5 were used in the least squares calculations to determine log K_3.

For titrations to determine log K_2 and log K_1, the usual NBS buffer calibration method was used to convert pH_{meas} to $p[H^+]$ (as described in Chapter 2). For the determination of log K_2, 8 titrations were performed with 3 separate stock ligand solutions (*ca.* 5 x 10^{-3} mol L^{-1}); the average number of datum points per titration was 21 (167 in total). Data in the $p[H^+]$ range 6.3 - 9.6 were used in the least-squares calculations to determine log K_2. To determine log K_1, 7 titrations were performed with 3 separate stock ligand solutions (*ca.* 5 x 10^{-3} mol L^{-1}). pH measurements become unreliable in highly alkaline solutions due to liquid junction effects, and only a limited number of data in the appropriate pH range were obtained with each titration. Therefore, data for 2 or 3 titrations on the same stock ligand solution were pooled and the least squares calculations were performed on these combined data sets (3 in total). There was an average of 20 datum points in each combined data set (61 in total). Data in the $p[H^+]$ range 9.5 - 10.9 were used in the least-squares calculations to determine log K_1.

To the author's knowledge, the equilibrium constant for the binding of K^+ to MHBS has not been determined; hence, this parameter could not be included in the calculations. Variations in ionic strength over the course of the titration were not considered; the change in charge on the ligand (-1.0 to +1.0) will not have a significant impact on the ionic strength.

Values for log K_n (n = 1 - 3) are given in Table 4.6.

Table 4.6: Protonation Constants for 5-Methoxy-N-(2-hydroxybenzyl)sarcosine: 25°C, I = 0.1 M KCl

Reaction	log K
$H^+ + L^{2-} \leftrightharpoons HL^-$	11.7 ± 0.1
$H^+ + HL^- \leftrightharpoons H_2L$	8.15 ± 0.03
$H^+ + H_2L \leftrightharpoons H_3L^+$	1.92 ± 0.06

Data are reported as mean ±3σ.

Cu(II)-MHBS Stability Constants

Four titrations on two separate metal-ligand solutions were performed (1.56 x 10^{-3} mol L^{-1} Cu(II), 1.62 x 10^{-3} mol L^{-1} MHBS). Data in the p[H^+] range 3.35 - 5.10 were used in the least squares refinement, with an average of 25 datum points per titration (99 in total). Because of the high uncertainty on K_1 for ligand protonation, MHBS was treated as a diprotic ligand (protonation of the phenoxide group was omitted). The values of the stability constants were: log K_{CuL} = 7.05 ± 0.04, log $K_{CuLH_{-1}}$ = -3.98 ± 0.03 (reported as mean ± 3σ for four titrations). The percentage formation of these species as a function of pH is shown in Figure 4.4.

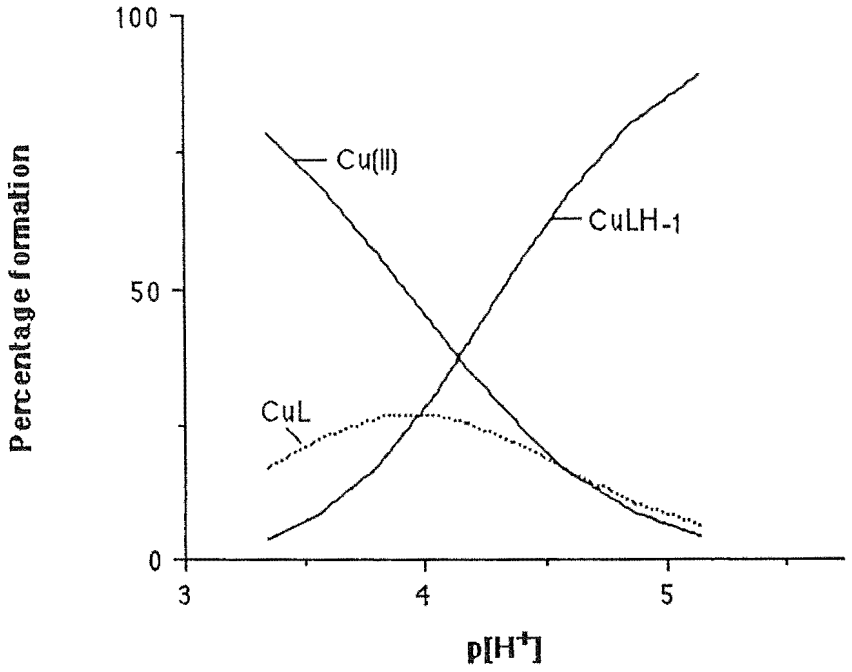


Figure 4.4: Species Distribution Diagram for the Cu(II)-MHBS system.

The UV-visible absorption spectra for MHBS and the 1:1 Cu(II)-MHBS solutions were measured over the pH range 4 - 11; the solutions were green above pH 3.0. In the presence of Cu(II) there was a new absorbance peak at 430 nm, the intensity of which increased from pH 4 - 6, and was then constant at pH 6 - 7 (ε_{max} = 500 L mol^{-1} cm^{-1}). At pH 11.0, this peak shifted to 410 nm (ε_{max} = 470 L mol^{-1} cm^{-1}). In the spectrum for the ligand there was a shoulder at 230 nm and an absorbance peak at 305 nm (ε_{max} = 3 800

L mol^{-1} cm^{-1}) in the pH range 4 - 6, and at 235 nm and 310 nm respectively at pH 13. These absorbances were also present in the spectra for the Cu(II)-MHBS solutions.

On titration of acidic 1:2 Cu(II)-MHBS solutions, a blue-violet precipitate formed at $p[H^+] \geq 5.0$ and precipitation was virtually quantitative. (The concentrations of Cu(II) used were 2.07×10^{-3}, 1.04×10^{-3}, and 0.78×10^{-3} mol L^{-1} with MHBS concentrations of 4.18×10^{-3}, 2.09×10^{-3}, and 1.60×10^{-3} mol L^{-1} respectively).

4.3 DISCUSSION

4.3.1 Standardization of KOH and HCl Solutions

Initially the KOH solutions were standardized by titration against weighed amounts of potassium hydrogen phthalate (BDH Analar, dried at 110°C, 1 - 2 h) and HCl solutions were standardized by titration against weighed amounts of sodium carbonate (BDH Analar, dried at 260 - 270°C, 30 min). After drying, the potassium hydrogen phthalate and sodium carbonate were stored in a desiccator over anhydrous $CaCl_2$.

However, problems were found with each of these standards. The standardized concentrations of HCl and KOH thus obtained were not internally consistent. That is, when HCl was titrated against KOH the end point did not correspond to the potassium hydrogen phthalate-determined concentration of KOH (the effective KOH concentration was *ca.* 0.5% less than this value).

By re-drying the potassium hydrogen phthalate sample it was established that it had not absorbed water on storage. According to Vogel (1974), potassium hydrogen phthalate is almost non-hygroscopic. Covington (1981) reported that there have been problems with the use of potassium hydrogen phthalate as an acidimetric standard with the commonest impurity being phthalic acid. Such an impurity would result in an apparently lower KOH concentration.

Titration against anhydrous sodium carbonate is one of the methods recommended by Vogel (1974) for the standardization of HCl. However, sodium carbonate is hygroscopic. Re-drying of a sodium carbonate sample established that the material had absorbed water (1% by weight) on storage.

Standardization of HCl and KOH with Tris and Tris.HCl respectively allowed internally consistent concentrations to be obtained; this method was therefore used for all measurements. The concentration of HCl determined by titration against Tris was *ca.* 0.4% less than that determined by sodium carbonate; the concentration of KOH determined by titration against Tris.HCl was *ca.* 0.3% less than that determined by potassium hydrogen phthalate.

4.3.2 Testing of the Apparatus and Verification of the Calculation Procedure used for the Determination of Stability Constants

IUPAC have reported a recommended procedure for testing the apparatus and technique used to determine metal-complex stability constants (Braibanti et al., 1987). This method involves the titration of nickel(II)-glycine mixtures in 1.0 mol L^{-1} NaCl. This system has been carefully studied by a number of different research laboratories; hence, very reliable stability constants (and standard deviations for these) are available for all the complex species present.

However, in the present work a constant ionic medium of 0.10 mol L^{-1} KCl was used; hence, nickel(II)-glycine was considered to be an inappropriate test system. Instead, the Cu(II)-histidine system was studied. The values of the stability constants determined in the present work (Table 4.1) established that the apparatus and technique used allowed reliable stability constants to be obtained. The estimate of precision for the IUPAC recommended stability constants (given in brackets in Table 4.1) refers to "the possible error in the last digit" (Pettit, 1984). With the exception of CuLOH (which has a maximum concentration of only 0.6% of TM), all the calculated constants agree with the IUPAC recommended values to within 3σ.

4.3.3 Al(III)-Malonic Acid Equilibria

Malonic Acid Protonation Constants

Although no data could be found which refer to the same ionic strength and medium as that used in the present work, the protonation constants reported for malonic acid in Table 4.2 are within the range of values reported by other workers (Table 4.7).

Table 4.7: Literature Values for Malonic Acid Protonation Constants

Reference	log K_1	log K_2
This work (excluding K^+ ion-pairing)	5.25 ± 0.05	2.60 ± 0.08
Marathe et al. (1984): 25°C, 0.2 M $NaClO_4$	5.27	2.56
Abdullah & Monk (1985): 25°C, 1.0 M NaClO4	5.061	2.67
Capone et al. (1985): 25°C, 0.09 M Et_4N	5.42	2.64
Smith et al. (1985): 25°C, ?	5.3	nr
Athavale et al. (1967): 30°C, 0.2 M $NaClO_4$	5.11	2.59
Dutt et al. (1976): 30°C, 0.1 M $NaClO_4$	5.45	2.80
Jackson & Cosgrove (1982): 37°C, 0.15 M NaCl	5.238	2.644
Daniele et al. (1983): 37°C, 0.15 M Et_4N	5.39	nr

nr = not reported.

It is of interest to compare the protonation constants for malonic acid with those for methylmalonic acid. Dutt et al. (1976) measured log K_1 = 5.61 and log K_2 = 3.26 for methylmalonic acid at 30°C in 0.1 mol L^{-1} $NaClO_4$ (c.f. Table 4.7). The electron donating methyl substituent results in methylmalonic acid being a weaker acid than is malonic acid. In 0.6 mol L^{-1} NaCl media at 25°C, Marklund & Öhman (1990) obtained log K_1 = 5.091 and log K_2 = 2.772 for methylmalonic acid.

Problems Associated With the Determination of Malonic Acid Protonation Constants

Some problems with ligand stoichiometry were encountered in the titration of malonic acid with standard KOH. Firstly, the difference between the concentration of ligand determined from the titration end point and that expected from the weight of ligand was much larger (3 - 6%) than that observed for the other ligands in this study. Secondly, when the concentration of ligand was allowed to vary in the least squares calculation, the value obtained was always significantly *greater* than that allowable by the acid concentration, TH/2, by 1 - 2.5%. (The least squares value of TL was, however, always less than that expected from the weight of ligand). This result was observed for 6 titrations on 2 separate stock ligand solutions. Fixing the value of TL at TH/2 in the least squares refinement resulted in a much poorer fit to the data (R-factors increased up to 10-fold and errors on the calculated constants were much larger). Further, when TL was fixed at TH/2 the calculated value of log K_1 was 0.01 - 0.03 log units greater, and that of log K_2 was 0.05 - 0.09 log units greater, than the values obtained when TL was a variable parameter.

The sample of malonic acid which was initially titrated in this work had been recrystallized by Gregor (1988, unpublished results) from refluxing benzene/2% ethanol. (Hamer et al. (1940) had purified malonic acid by recrystallization from a mixture of benzene and ether containing 5% petroleum ether). A second sample of malonic acid was recrystallized from refluxing 'Spectroscopic' ethanol (BDH) in the present work; (the ligand was found to be insoluble in benzene/ethanol solution). However, the least squares refined value of TL was again greater than that allowable from the titration end point for titrations on this ethanol-recrystallized malonic acid. Although it is likely that some decomposition of the malonic acid occurred under the recrystallization conditions used (b.pt ethanol = 78.5°C; CRC Handbook of Chemistry and Physics), any decomposition products should not have been isolated with the malonic acid fraction (Dr P.J. Steel, pers. comm. 1990).

Several workers have made potentiometric measurements on unpurified malonic acid samples (e.g. Daniele et al., 1982; Marathe et al., 1984; Capone et al., 1985). The malonic acid used in the present work was supplied by Riedel-de Haën and, according to the manufacturer, was 99% pure. Some titrations were performed on this unpurified material. In

this case, the total acid determined by the titration end point was 0.6% less than that expected from the weight of ligand used and, importantly, the least squares refined value of TL was equal to TH/2. The protonation constants calculated from least squares analysis of the unpurified malonic acid titration data were the same as those obtained for the recrystallized sample when the concentration of ligand was fixed at TH/2.

Attempts were made to trace the source of this discrepancy between TH and TL in the recrystallized malonic acid samples. Two perspectives were considered, *viz:* the presence of decomposition products arising from the recrystallization procedure, and the presence of alkaline metal impurities (e.g. Na_2malonate which would lower the number of titratable protons).

Attempts to Detect Decomposition Products in Recrystallized Malonic Acid

The melting point of a compound is a good test of its purity. A 1% impurity could effect a 5 - 10°C decrease in melting point (Dr P.J. Steel, pers. comm., 1990). The melting point of the unpurified malonic acid sample was 135 - 138°C, that of the sample recrystallized by Gregor was 135 - 138°C, and that of the sample recrystallized in the present work was 136°C. Hence, melting point measurements provided no evidence for impurities in the recrystallized malonic acid samples.

^{13}C and ^{1}H nuclear magnetic resonance spectra did not detect any extraneous compounds in the malonic acid samples.

The calculated percent composition of malonic acid (assuming no associated water molecules) is C, 34.6% and H, 3.85%. The sample recrystallized by Gregor contained C, 34.58% and H, 3.77%; the sample recrystallized in the present work contained C, 34.60% and H, 4.02%. Hence, microanalysis provided no evidence for impurities in the recrystallized malonic acid samples.

Acetic acid will be a decomposition product of malonic acid. The possibility that acetic acid was contributing to the malonic acid titrations was considered. Inclusion of a fixed amount of acetic acid (1 - 5% of the malonic acid concentration) in the least squares calculations provided no improvement in the fit to the data; the ORGLS program would not

refine the concentration of acetic acid if it was allowed to be a variable parameter. Analysis of the titration data *via* SUPERQUAD (kindly performed by Dr J.E. Gregor) also provided no evidence for the presence of acetic acid, i.e. the protonation constants for acetic acid were included in the calculation, and when the concentration of acetic acid was allowed to vary during the least squares refinement this parameter tended to zero.

A method for detecting the presence of impurities ('dirt acid') in the titration of a protic ligand is to plot log X *versus* X/(1+X) for a hypothetical curve and for the $\bar{n}_{obs}$ curve. (Where $X = K_1 [H^+]$ and $\bar{n} = X/(1+X)$.) The shapes of these curves are then compared. A discrepancy at $\bar{n} = 0.5$ indicates the presence of a protic impurity; a discrepancy at $\bar{n} = 1.0$ indicates an error in ligand stoichiometry. For a titration of the recrystallized malonic acid, the curves for $\bar{n}_{obs}$ and $\bar{n}_{calc}$ *versus* log K.$[H^+]$ were superimposable.

Therefore, all the aforementioned evidence strongly suggests that there is no impurity in the recrystallized malonic acid samples.

Attempts to Detect Alkali Metal Impurities in Recrystallized Malonic Acid

The observation that the least squares refined value of TL for the recrystallized malonic acid was greater than TH/2 may indicate the presence of an alkali metal impurity, such as Na_2malonate. The sodium and potassium content of a solution of recrystallized malonic acid was measured by flame-emission atomic spectroscopy; calcium was measured by flame atomic absorption spectroscopy. There was no detectable potassium or calcium. A sodium content of 0.14 weight% was measured; however, this amount is not sufficient to account for the observed discrepancy.

Another possibility is that the malonic acid forms complexes with silica from the glass storage container. For example, oxalic acid has been reported to form a stable complex with silicate ions at neutral pH (Marley et al., 1989). The silica content of a malonic acid solution which had been stored in a glass volumetric flask at 6°C for 2 months was measured using the "Heteropoly Blue Method" (Hach Field Test Kit). Assuming that the silica is present as SiO_3^{2-}, then the measured silica content of the malonic acid solution corresponded to 0.2% of the malonic acid concentration (on a molar basis). Again, the magnitude of this impurity is

not sufficient to account for the observed discrepancy between TH and TL (1 - 2.5%) in the titrations of recrystallized malonic acid.

In conclusion, recrystallization of the malonic acid caused a ligand stoichiometry problem. Despite a considerable investment of time the nature of this effect could not be established. Therefore, for solution equilibria studies it is recommended that malonic acid should not be further purified before use.

Al(III)-Malonic Acid Stability Constants

Nonrecrystallized malonic acid was used for the 4 titrations from which the stability constants were finally calculated. It was established that equilibrium in the Al(III)-malonic acid system was attained rapidly (within 5 min) after each addition of alkali. To minimize localized high concentrations of alkali (and subsequent slow dissolution of hydroxy species) KOH titrant was added at the slowest rate allowable by the automatic titration system (0.125 mL min^{-1}).

The ñ/pL plots for different metal:ligand ratios were coincident for data in the $p[H^+]$ range 2.2 - 5.0 (Figure 4.1). For data above $p[H^+]$ 5.0 the ñ/pL plots showed some deviation from a coincident family of curves, suggesting the formation of hydroxy species.

The plateau in the Bjerrum plots at ñ = 2.0 indicates the formation of AlL_2 as a dominant species. Marklund and Öhman (1990) reported that ñ reached a limiting value of 3.0 for the Al(III)-methylmalonic acid system. However, only use of a 1:10 metal:ligand ratio allowed these authors to obtain data with ñ values greater than 2.5. In the present work, ñ values up to 2.4 were obtained for titrations at a 1:3 and 1:5 ratio, suggesting that a weak complex, AlL_3, was also formed.

The initial least squares calculations were therefore performed with the stability constants for AlL, AlL_2, and AlL_3 as the parameters to be refined. This model provided a good fit to the data. However, for data in the $p[H^+]$ range 5.0 - 5.5 there was a systematic trend in the residuals in the least squares refinement; the total acidity calculated by the model was greater than that observed. This effect was particularly pronounced for the 1:2 titration data, for which the region of poor fit was $p[H^+]$ 4.3 - 5.5.

Inclusion of AlL_2OH in the model significantly improved the fit to the data in the higher p[H^+] region. It is noted that the presence of this species could be doubtful; less than 3% formation was calculated at p[H^+] 5.5 for a 1:3 and a 1:5 ratio. Only 5% formation was calculated for a 1:2 ratio at p[H^+] 5.5; under these conditions AlL_3 is not a competing species. The end point for the 1:2 titration was slightly greater than that corresponding to the titration of 2.0 protons per ligand plus acid added to the system; this provides some additional evidence for the existence of AlL_2OH. Further, the existence of AlL_2OH is 'chemically reasonable'; it is formed in a p[H^+] region in which the precursor for its formation, AlL_2, is present in significant amounts (Figure 4.2). Further, log K (AlL_2OH) is greater than that for the first hydrolysis reaction of Al(III).

The species AlLOH was also considered. Although the model refined when this parameter was included (and the residuals in the least squares refinement were improved), it was calculated to form in a p[H^+] region in which no significant amount of AlL remained in solution. Therefore, formation of AlLOH was not considered chemically reasonable.

Pósci and Fábián (1988) studied the Ti(III)-malonic acid system by pH potentiometric titration (25°C, I = 0.1 M KCl). (Ti(III) is a more strongly hydrolyzed metal ion than is Al(III).) These authors titrated solutions having metal-to-ligand ratios in the range 1:2.4 to 1:6. No evidence for hydroxy complexes was observed. However, the pH range over which the titration data were analyzed (*via* PSEQUAD) was not stated. The equilibrium model which best described complexation in the Ti(III)-malonic acid system was: TiL (log K = 6.83); TiL_2 (4.99); and, TiL_3 (2.84) (Pósci & Fábián, 1988). These constants are similar to those obtained in the present work for the coordination of Al(III) to malonic acid.

Previous Studies

Values of the stability constants for Al(III)-malonic acid complexation reported by other workers are compared with those calculated in the present work in Table 4.8.

Table 4.8: Literature Values for Al(III)-Malonic Acid Stability Constants

Species	log K[a]	log K[b]	log K[c]	log K[d]
AlL	6.71 ± 0.03	5.24	6.15	6.264
AlL_2	4.94 ± 0.02	4.16	3.95	4.847
AlL_3	2.61 ± 0.04	nr	nr	2.189
AlL_2OH	-7.0 ± 0.1	nr	nr	nr

nr = not reported.

[a]present work.

[b]Athavale et al. (1967); 0.2 mol L^{-1} $NaClO_4$, 30°C.

[c]Dutt et al. (1976); 0.1 mol L^{-1} $NaClO_4$, 30°C.

[d]Jackson & Cosgrove (1982); 0.15 mol L^{-1} NaCl, 37°C.

There is obviously poor agreement between these constants! Athavale et al. (1967) did not use a least squares refinement process to determine their reported stability constants (linear plots of log (1-ñ)/ñ *versus* pL were used); hence their values may not be of very high accuracy. Dutt et al. (1976) also used graphical methods for the determination of stability constants.

Jackson and Cosgrove (1982) analyzed their titration data *via* the least squares refinement program MINIQUAD. They titrated Al(III)-malonic acid solutions at 1:2.3, 1:2.05, and 1:1.5 metal:ligand ratios (using data in the p[H^+] range 2.0 - 4.6; surprisingly they claimed a $\tilde{n}_{max}$ = 2.3 for a 1:2.3 solution). Further, in contrast to the present work, Jackson and Cosgrove (1982) reported that the kinetics of complexation of Al(III) by malonic acid was slow; hence, "long" delay times between additions of alkali were required for the system to attain equilibrium. The actual delay time used by these authors was not stated.

By use of nonlinear least squares refinement on titration data at 1:2, 1:3 and 1:5 Al(III):malonic acid ratios, the present work has established constants for AlL, AlL_2 and

AlL_3 with a high degree of precision. Evidence was also obtained for formation of AlL_2OH as a minor component; this species has not previously been proposed for this system.

4.3.4 Problems Encountered in Al(III)-Malonic Acid Studies

A large number (96) of titrations were performed on the Al(III)-malonic acid system for which the ñ/pL plots were disparate. Over a period of time (2 yr) several problems were isolated and conditions were finally established which provided the family of ñ/pL plots from which the reported stability constants were obtained.

A *considerable* amount of time was invested in isolating the problems associated with this system; these are now discussed.

Use of an Aged Al(III) Stock Solution

Initially, titrations were performed using a stock aluminium(III) solution which had been prepared by a previous worker (Kennedy, 1984) and stored in a polyethylene container at 6°C; (the acid and Al(III) content were firstly re-determined). Bjerrum plots for titrations of Al(III)-malonic acid solutions prepared from this stock solution were not reproducible (i.e. disparate ñ/pL plots were obtained for replicate titrations on the same metal-ligand solution). Further, ñ values greater than 3.0 were obtained. The cause of these problems could not be traced. It is possible that extensive hydrolysis of the Al(III) solution had occurred slowly over time (5 yr), although this does not seem likely in acidic solution (*ca.* 0.1 mol L^{-1} HCl). Alternatively, the solution may have become contaminated at some stage in its history.

Two fresh stock Al(III) solutions were prepared (from the same solid sample of AR $AlCl_3.6H_2O$ from which the original Al(III) solution had been prepared). Use of these Al(III) solutions improved the agreement between ñ/pL plots for replicate titrations. However, the measured acid content of these solutions was greater than that corresponding to the amount of acid added to suppress Al(III) hydrolysis. A much purer Al(III) sample was purchased in the latter stages of this work (ALFA, 99.9995%). The acid content of a solution prepared

from this sample *was* consistent with the amount of acid added. This purer Al(III) solution was used for the 4 titrations from which the stability constants were calculated.

Solution Composition Uncertainties

For the initial titrations the contribution of solution composition uncertainties to discrepancies between ñ/pL plots was considered. Despite careful analysis, no specific solution stoichiometry errors could be isolated. All stock solutions used to prepare the metal-ligand solutions were standardized separately (and reproducibly), i.e. the stock Al(III), malonic acid, and HCl solutions. To minimize any solution composition errors, some of the initial titrations were performed with no added HCl. However, this did not improve the agreement between ñ/pL plots for different metal:ligand ratios (but these data *were* in agreement with the titrations which did contain added HCl).

pH Calibration Problems

Problems with the stability of pH measurements were encountered in the initial titrations. The pH readings obtained for the NBS buffer solutions required *ca.* 30 min to reach stable values, and the change in the pH measured for these buffers, before as compared with after each titration, was typically 0.01 - 0.03 pH units (the time required to complete each titration was *ca.* 3 - 6 h). The drift in pH readings for the buffer solutions was uniform, i.e. a similar change was observed in the measured pH of all three NBS buffers. In converting the measured pH values for the Al(III)-malonic acid titrations to $p[H^+]$ values, the change in NBS buffer calibrations was assumed to have been linear with time over the course of the titration.

It was thought that the problems with stability of pH readings were related to the calomel reference electrode (five glass electrodes, Beckman (E-2 membrane, low sodium error) and Radiometer (G202B), were tested and all showed similar response characteristics). Many attempts were made to improve the performance of the calomel electrodes (Radiometer K401 and Beckman 39417), but only limited success was achieved. In the latter stages of this work Russell glass (SWR757) and calomel reference (CR5)

electrodes were purchased; these had far superior response characteristics. The Russell electrodes were used for the 4 titrations from which the stability constants were calculated.

Conclusion

Use of a very pure Al(III) solution and stable electrodes enabled reproducible data to be obtained.

4.3.5 Al(III)-Isocitric Acid Equilibria

Isocitric Acid Protonation Constants

The protonation constants for isocitric acid are given in Table 4.4. To the author's knowledge, these constants have not been reported by any other workers. Inclusion of K^+L^{3-} ion pairing lead to an increase of 0.20 in log K_1. (To the author's knowledge, the stability constant for K^+-isocitrate ion pairing has not been reported in the literature; therefore, the value for citric acid was assumed to be valid: log K = 0.77 (Rechnitz & Zamochnick; 1964)). A similar effect was reported for citric acid by Gregor and Powell (1986b).

For titrations at low ionic strength ($\leq$ 0.1 mol L^{-1}) involving polyvalent ions, significant changes in ionic strength can occur over the course of the titration as the polyvalent ion is formed. Correcting the data for the variation in ionic strength which occurs throughout the titration of isocitric acid did not significantly change the calculated protonation constants (Table 4.4).

It is of interest to compare the protonation constants for isocitric acid with those for citric acid (Table 4.9). Comparison of the protonation constants for isocitric acid with those for citric acid (determined by Gregor and Powell (1986b)) indicates that log K_1 and log K_2 for these ligands are not significantly different to within 3σ. However, H_2L^- for isocitric acid is significantly more basic, i.e. log K_3 as measured is greater, than the value Gregor and Powell (1986b) reported for citric acid. There is no apparent chemical reason why this should be the case.

Table 4.9: Comparison of Protonation Constants for Citric and Isocitric Acid: 25°C, I = 0.1 M KCl

Reaction	Constant	Isocitric Acid[a]	Citric Acid[b]	Citric Acid[c]
$H^+ + L^{3-} \leftrightharpoons HL^{2-}$	$\log K_1$	5.88 ± 0.04	5.90 ± 0.03	5.92 ± 0.03
$H^+ + HL^{2-} \leftrightharpoons H_2L^-$	$\log K_2$	4.29 ± 0.05	4.47 ± 0.01	4.35 ± 0.03
$H^+ + H_2L^- \leftrightharpoons H_3L$	$\log K_3$	3.09 ± 0.05	3.20 ± 0.03	2.91 ± 0.09

All data include K^+L^{3-} ion pairing and were corrected for variation in ionic strength.

[a]Determined in the present work; mean ± 3σ for 5 titrations.

[b]Determined in the present work; mean ± 3σ for 2 titrations.

[c]Gregor and Powell (1986b); mean ± 3σ for 4 titrations.

The value for log K_3 indicates a high acidity; therefore determination of this constant is subject to the most error. It will also be very dependent on the method used to calibrate the glass electrode as a hydrogen ion concentration probe. In both the present work and for the data reported by Gregor and Powell (1986b) a combination of *o*-phthalic acid titrations and HCl-KOH titrations (over a similar p[H^+] range) was used to calibrate the glass electrode. However, in contrast to the present work, Gregor and Powell (1986b) calculated a *linear* regression line through their combined *o*-phthalic acid and HCl titration data. As discussed in Chapter 2, H_3O^+ makes a significant contribution to the electrode liquid junction potential at low pH; hence, a linear correction in [H^+] may be a poor approximation. To test the effect of electrode calibration on the calculated stability constants, two titrations on a citric acid solution (2.5×10^{-3} mol L^{-1}) were performed in the present work; results are given in Table 4.9. The value of log K_3 is significantly different from that reported by Gregor and Powell (1986b). This result highlights the critical dependence of stability constants on the electrode calibration technique employed.

Log K_3 for citrate and isocitrate indicates a higher acidity than that for tricarballylic acid (log K_3 = 3.47; Campi et al., 1964) due to the electron withdrawing effect of the hydroxyl group.

Al(III)-Isocitric Acid Stability Constants

The Al(III)-isocitrate system was studied to further elucidate the coordination of citrate to Al(III) which forms an exceptionally stable complex (Gregor & Powell, 1986b; Öhman, 1988). Gregor and Powell (1986b) proposed that the stability of the 1:1 Al(III)-citrate complex resulted from intramolecular hydrogen bonding between the non-coordinating carboxyl group and the coordinated C(2)-OH group. A parallel interaction cannot occur for isocitrate. Formation of a less stable AlL complex in the Al(III)-isocitrate system would therefore provide supporting evidence for hydrogen bonding stabilization in the Al(III)-citrate system. To the author's knowledge, quantitative analysis of complexation of Al(III) by isocitrate has not previously been reported.

Recently, Öhman (1988) has demonstrated the extremely slow kinetics of complexation in the Al(III)-citrate system (20 h required for equilibrium to be attained). This result was confirmed in the present work (by rapid addition of KOH to Al(III)-citrate solutions (5×10^{-3} mol L^{-1} in both Al(III) and citrate)). At pH 4 drift to more acidic values occurred (by 0.37 over 20 h); at pH 7.5 the pH drifted to more alkaline values (by 0.48 over 20 h). The magnitude of this drift is *ca.* half that reported by Öhman (1988); however, the concentrations used in the present work were approximately half those used by Öhman (1988).

In contrast, the Al(III)-isocitrate solutions attained equilibrium within 1 h below pH 6. This observation is interesting and may indicate a different stoichiometry at each pH from that in the Al(III)-citrate system. Although the magnitude of the drift was apparently less in the isocitrate system, a very rapid change in pH could have occurred immediately after mixing Al(III) and isocitrate. This more rapid equilibration allowed the stability constants for complexation of Al(III) by isocitrate to be calculated from direct titrations, with a 30 min delay time at each datum point (whereas Öhman (1988) used a batch technique for Al(III)-citrate).

For these equilibration measurements, the addition of aliquots of KOH was performed under rapid stirring to minimize localized high concentrations of alkali. If slow dissolution of hydroxy species was occurring then the drift would have been in the opposite direction to that observed.

For the Al(III)-citrate system, Öhman (1988) reported log $\beta_{-3,1,1}$ = -4.92 (i.e. for the reaction: $Al^{3+} + H_3L \leftrightharpoons AlL + 3H^+$); a value of -5.06 was reported by Gregor and Powell (1986b).

In contrast, log $\beta_{-3,1,1}$ = -6.3 was calculated for Al(III)-isocitrate in the present work, indicating a complex of significantly lower stability. This result provides supporting evidence for hydrogen bonding contributing to complex stability in the Al(III)-citrate system. Evidence for the existence of such an interaction is discussed by Gregor and Powell (1986b). These authors stated that there was no evidence for hydrogen bonding contributing to the acidity of the free citrate ligand (if it did, then hydrogen bonding could not be invoked to explain the high stability of the 1:1 Al(III)-citrate complex). In support of this argument, the protonation constants determined for isocitrate in the present work are not measurably different from those for citrate (Table 4.9).

The $AlHL^+$ complex in the isocitrate system (log $\beta_{-2,1,1}$ = -3.19) is also less stable than that for citrate (log $\beta_{-2,1,1}$ = -2.68; Öhman, 1988).

The end point stoichiometry observed for Al(III)-isocitrate was the same as that reported for Al(III)-citrate (Öhman, 1988), indicating $Al_3(OH)(LH_{-1})_3^{4-}$ as the terminal species. However, preliminary calculations on the Al(III)-isocitrate titration data above pH 4 indicated that the system was not well described by Öhman's (1988) model for the Al(III)-citrate system. This observation may be consistent with the more rapid attainment of equilibrium in the Al(III)-isocitrate system.

4.3.6 Cu(II)-Tetrabutanoic Acid Equilibria

Tetrabutanoic Acid Protonation Constants

The protonation constants for tetrabutanoic acid are reported in Table 4.5. The same constants were calculated for both unrecrystallized ligand and that recrystallized from ethanol. Inclusion of a correction for variation in ionic strength throughout the titration effected a small change in the mean value of log K_4 and there was a slight improvement in the least squares fit. The magnitude of this effect will increase as the concentration of ligand is increased; it will be less apparent at higher concentrations of background electrolyte. Therefore, as recommended by Gregor and Powell (1986b), such ionic strength corrections should be taken into account when polyprotic ligands are titrated in low concentrations of background electrolyte.

To the author's knowledge, the only other protonation constants reported for tetrabutanoic acid were measured by Purdie et al. (1972); Table 4.5. These authors reported thermodynamic constants which are valid at zero ionic strength (and 25°C) and do not require correction for ion-pairing interactions. In contrast, the values determined in the present work are concentration quotients. Inclusion of ion-pairing interactions is expected to increase log K_1 by only *ca.* 0.2 log units (by comparison with malonate and isocitrate). Therefore, there is a large discrepancy between the values obtained in the present work and those reported by Purdie et al. (1972).

It is of interest to note that the values for the protonation constants of citric acid obtained by Purdie et al. (1972) (log K_1 = 6.40, log K_2 = 4.76, log K_3 = 3.13) are not in good agreement with those reported by Gregor and Powell (1986b) (Table 4.9) and many other workers.

The protonation constants for tricarballylic acid (log K_1 = 5.89, log K_2 = 4.54, log K_3 = 3.47; Campi et al., 1964) indicate a higher basicity than the values determined for tetrabutanoic acid in the present work (but are similar to the values reported by Purdie et al. (1972)). The electron withdrawing properties of the additional carboxyl group in

tetrabutanoic acid would be expected to lower all log K_i values. This throws serious doubt on the log K_i values (i = 2 - 4) reported by Purdie et al. (1972) for tetrabutanoic acid.

Cu(II)-Tetrabutanoic Acid Stability Constants

Precipitation occurred on titration of Cu(II)-tetrabutanoic acid solutions. Therefore, the stability constants for this system could not be determined. No experimental conditions could be found which prevented precipitate formation. The precipitate formed at $p[H^+]$ 3.5 could be the nonionic species CuH_2L. It was reasoned that by $p[H^+]$ *ca.* 5.0, the coordinated ligand will deprotonate to generate the $CuHL^-$ species, which should be soluble. Hence, to minimize the chances of precipitate formation, conditions which promote the formation of $CuHL^-$ were considered, i.e. high concentrations of ligand and/or low concentrations of metal. However, precipitation occurred even in a 1:5 metal:ligand solution.

Practically, the lowest concentrations of metal and ligand which can be used for the determination of the stability constants for this system are 5×10^{-4} mol L^{-1} and 1×10^{-3} mol L^{-1} respectively. If a solution of this composition does not precipitate at pH > 5 then it should be possible to collect data to determine the stability constants by starting the titrations at high pH, then adding acid until the precipitation boundary is reached. However, precipitation occurred in a solution of the above composition at $p[H^+] = 5.0$. At this pH, in the absence of Cu(II), about 40% of the ligand is dissociated into H_2L^{2-} and about 40% is present as HL^{3-}. Therefore, a significant amount of the insoluble CuH_2L species could still be present at pH 5. In addition to CuH_2L, it is possible that insoluble Cu(II)-tetrabutanoate polymers form at high pH. (The ligand itself was completely soluble at all pH values.)

The stability constants for Cu(II) complexation by the structurally similar ligand tricarballylic acid have been reported (Campi et al., 1964). The species formed in the Cu(II)-tricarballylic acid system were CuH_2L^+, $CuHL$, CuL^-, and Cu_2L^+ (Campi et al., 1964). These authors found no evidence for the formation of species of the type $Cu(H_nL)_m$, with m > 1, under their experimental conditions (2×10^{-3} mol L^{-1} tricarballylic acid, 5×10^{-4} to 2×10^{-2} mol L^{-1} Cu(II); 0.10 mol L^{-1} $NaClO_4$, 20°C).

4.3.7 Cu(II)-5-Methoxy-N-(2-Hydroxybenzyl)Sarcosine (MHBS) Equilibria

MHBS Protonation Constants

The protonation constants for MHBS are given in Table 4.6. To the author's knowledge, these constants have not been reported by any other workers. Log K_1 (11.7) corresponds to protonation of the phenoxide group; log K_2 (8.15) protonation of the amino group, and log K_3 (1.92) represents protonation of the carboxylate group. Log K_1 for MHBS is remarkably high compared with a value of 9.79 reported for phenol (McBryde, 1968). This could arise from intramolecular hydrogen bonding between the phenolic hydroxyl group and the amino nitrogen. Supporting evidence for this proposal is provided by the protonation constants for hydroxy and methoxy substituted benzylamines. The presence of an amine and a methoxy substituent increases the basicity of the phenoxide group. Log K_1 for 3-hydroxy-2-methoxy benzylamine is 10.54; that for the 4-hydroxy-3-methoxy derivative is 10.5 (Perrin, 1965). For a phenolic hydroxyl group adjacent to an amine group, as in 2-hydroxy-3-methoxy benzylamine, the phenoxide protonation constant was further increased to 11.06 (Perrin, 1965).

Protonation constants for the related ligands glycine, N-methyl glycine (sarcosine), N,N-dimethyl glycine, and phenyl glycine are given in Table 4.10.

Log K for the protonation of the amino group in MHBS is about 1.6 - 2.0 log units lower than that for the ligands in Table 4.10. This is due to the influence of the electron withdrawing 5-methoxy-hydroxybenzyl group attached to N. The protonation constants for MHBS determined in the present work are in good agreement with those reported for the related ligand N,N'-bis(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic acid (HBED) (Taliaferro et al., 1984). The structure of HBED and its protonation constants (measured in 0.1 mol L^{-1} KCl at 25°C) are given in Figure 4.5.

Table 4.10: Protonation Constants for L-amino acids

Ligand	log K_1	log K_2
Glycine	9.72[a]	2.49[a]
	9.61[b]	2.40[b]
Sarcosine	10.16[a]	2.29[a]
	10.05[b]	2.22[b]
	10.14[d]	2.28[d]
N,N-dimethyl glycine	9.88[a]	2.14[a]
	9.77[c]	1.90[c]
Phenyl glycine	9.14[b]	1.61[b]

[a]Lim (1978), 0.5 mol L^{-1} KNO_3, 25°C.

[b]Lomozik (1984), 0.1 mol L^{-1} $NaClO_4$, 21°C.

[c]Lomozik & Wojciechowska (1985), 0.1 mol L^{-1} $NaClO_4$, 21°C.

[d]Debreczeni et al. (1983), 1 mol L^{-1} KCl, 25°C.

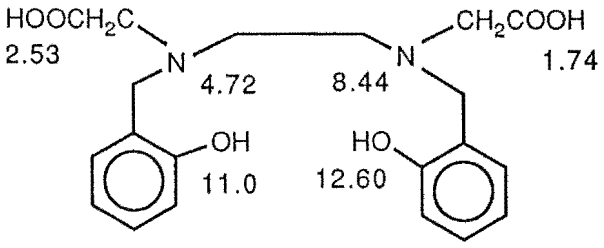


Figure 4.5: Structure and Protonation Constants of HBED

Cu(II)-MHBS Stability Constants

The titration end point was consistent with formation of $CuLH_{-1}$ (and CuL). When the stability constants for these two species were the parameters to be refined in the least

squares calculations there was a good fit to the data with no remaining systematic trends in residuals.

The UV-visible spectra indicated a shift in λ_{max} from 430 nm at pH 6 - 7 to 410 nm at pH 11.0. Because phenolate binding occurred at pH < 7, this shift is consistent with deprotonation of water bound to Cu(II). This absorbance peak probably corresponds to Cu(II)-phenolate binding (a charge transfer band). According to Hefford and Pettit (1981), complexation of phenol groups with Cu(II) results in an absorbance maxima between 375 and 405 nm (ε_{max} *ca.* 200 L mol^{-1} cm^{-1}). The increase in intensity of the peak at 430 nm on increasing the pH from 4 to 6 is consistent with $CuLH_{-1}$ formation occurring in this region of the titration.

The blue-violet precipitate which formed in the 1:2 Cu(II)-MHBS solutions was thought to be due to the CuL_2 or $Cu(LH_{-1})_2$ species. The blue-violet colour is consistent with coordination of two nitrogen atoms to Cu(II). Attempts were made to measure the stability constant for the CuL_2 and $Cu(LH_{-1})_2$ species. Ideally, the experimental conditions should be such that enough of the complex is formed to allow its stability constant to be determined, but not enough for precipitation to occur. A solution containing a 15% excess of ligand over Cu(II) was titrated (1.76 x 10^{-3} mol L^{-1} Cu(II), 2.05 x 10^{-3} mol L^{-1} MHBS). No precipitation occurred in this solution. However, the least squares calculation would not refine if CuL_2 or $Cu(LH_{-1})_2$ were included in the equilibrium model. This indicated that at pH > 4, $CuLH_{-1}$ is the dominant species in solution (and thus CuL_2 or $Cu(LH_{-1})_2$ is unable to form).

An attempt was made to include CuL_2 and $Cu(LH_{-1})_2$ as species in the least squares calculation on the 1:2 titration data (collected before precipitation began). For the solution containing 4.18 x 10^{-3} mol L^{-1} MHBS, precipitation began at p[H$^+$] *ca.* 4.1 and for those containing 2.09 x 10^{-3} and 1.6 x 10^{-3} mol L^{-1} MHBS, precipitation began at p[H$^+$] *ca.* 5.0 - 5.5. Again, the calculation would not refine if CuL_2 or $Cu(LH_{-1})_2$ was included in the equilibrium model. The 1:2 titration data were adequately described by considering formation of only CuL and $CuLH_{-1}$, i.e. there were no remaining systematic trends in residuals. This

observation indicates that, if the blue-violet precipitate does correspond to a CuL_2 or a $Cu(LH_{-1})_2$ species, then it must be very insoluble.

This system was studied to investigate a ligand in which enhanced coordination of a weakly binding group (phenolic hydroxyl) may occur by virtue of its proximity to a stronger donor site (an amino acid moiety). The values of the stability constants for Cu(II) complexation by MHBS indicate that such a process of 'cascade binding' does occur with this ligand, with the pK_a for deprotonation of the phenolic hydroxyl group being lowered to 3.98 in the presence of Cu(II).

A ligand which is closely related to MHBS is HBED (Figure 4.5). Cu(II) complexation by this ligand has been studied by L'Eplattenier et al. (1967). Coordination of the HBED phenolic hydroxyl groups to Cu(II) occurred at pH >5 and generated an absorbance peak at 385 nm (ε_{max} = 6 750). For HBED, log K ($CuH_2L \leftrightharpoons CuHL + H^+$) was -7.55 (calculated from data reported by L'Eplattenier et al., 1967). For this ligand, formation of a chelate ring on coordination with Cu(II) will involve the more weakly coordinating trans sites; whereas for MHBS, the coordination is into the more strongly binding square planar positions.

Implications for Cu(II) Complexation by Humic Substances

As noted in Section 4.1, a study of the Cu(II)-MHBS system was intended to elucidate the observed 'shortfall' in the Cu(II) binding strength of model ligands as compared to humic substances. The stability constants determined for the Cu(II)-MHBS system support the hypothesis that cascade binding by 2-hydroxybenzyl moieties attached to a strong primary coordination site could contribute to Cu(II) binding by humic substances. Attempts to model Cu(II) binding curves for humic substances including MHBS as a model ligand are discussed in Chapter 6.

A 6-membered chelate ring is formed on coordination of Cu(II) to MHBS. Ideally, (for reasons discussed by Gregor, Powell & Town, 1989a,b) it was desirable to study a ligand which formed a 7-membered chelate ring on coordination to Cu(II). However, to the author's knowledge a method to synthesize such a ligand was not available in the literature. The

magnitude of the cascade binding effect exhibited by MHBS is more dramatic than that which would be expected for a ligand which forms a 7-membered chelate ring.

CHAPTER 5

STUDIES ON THE AGGREGATION AND FRACTIONATION OF HUMIC SUBSTANCES

5.1 INTRODUCTION

Humic acids are heterogeneous, polydisperse compounds which are operationally defined on the basis of solubility. Even the average molecular weight of humic acid is the subject of debate; the reported value varies with the measurement technique, the pH, and the ionic strength of the solution. Much of this discrepancy may arise from the tendency of humic acid to form aggregates in solution with the degree of aggregation varying with pH, ionic strength, the type of counter ions, and the concentration of the sample. The predominant mechanism of aggregation for humic molecules is thought to be hydrogen bonding. For example, methylation of ionic functional groups lowers the apparent molecular size of humic acid moieties (Bartle et al., 1987).

The techniques which have been used to probe the molecular size or weight of humic acid include: gel permeation chromatography (Swift & Posner, 1971), ultrafiltration (Buffle et al., 1978b), vapour pressure osmometry (Aiken & Malcolm, 1987; Marinsky & Reddy, 1990), viscometry (Ghosh & Schnitzer, 1980), ultracentrifugation (Reid et al., 1990), small angle x-ray scattering (Wershaw & Pinckney, 1973b; Thurman et al., 1982), and flow field-flow fractionation (Beckett et al., 1987).

Information about the aggregation properties of humic acid may facilitate further understanding of its interactions with metal ions and hydrophobic species. It may also assist in designing an extraction scheme for humic acid which isolates a 'representative' sample of this humic fraction.

5.1.1 Scope of This Work

Gel permeation chromatography was used to probe the apparent molecular size distribution of humic substances. The molecular size distribution determined by this

technique is thought to be representative of aggregated humic acid structures rather than of individual molecules (Orlov et al., 1975). Conditions were developed which minimized adsorption of humic substances on the Sephadex gel matrix.

The aggregation of humic acid was studied as a function of pH in various media, and by equilibrium dialysis, with the associated changes in apparent molecular size being monitored by gel permeation chromatography.

XAD resins have been applied to the extraction of aquatic humic and fulvic acids, and soil fulvic acids. Their utility for isolation of humic acids extracted from soil was investigated. Gel permeation chromatography established that the large humic molecules were not significantly adsorbed by XAD resins.

5.2 EXPERIMENTAL

5.2.1 Electrolyte Solutions

Preparation of KNO_3, HCl, KOH, and borax buffer solutions was described in Chapter 3.

Synthetic seawater was prepared by dissolution of the appropriate amounts of AR salts in Milli-Q water to give a composition of: chloride, 0.535 mol L^{-1}; sodium, 0.459 mol L^{-1}; magnesium, 0.0523 mol L^{-1}; sulphate, 0.0276 mol L^{-1}; calcium, 0.01 mol L^{-1}; and potassium, 0.0097 mol L^{-1}.

Pyrophosphate solutions were prepared from $Na_4P_2O_7.10H_2O$ (Riedel-de Haën, für Analyse).

5.2.2 Gel Permeation Chromatography

Gel

Sephadex G-100, bead diameter 40 - 120 μm, (Aldrich) is a hydrophilic dextran with a small degree of epichlorohydrin cross-linking. According to the manufacturer, it

fractionates peptides and globular proteins in the molecular weight range 4 000 - 150 000 Dalton, and dextrans in the molecular weight range 1 000 - 100 000 Dalton.

Packing and Calibration of the Gel Column

Sephadex G-100 was swollen in the eluent (0.01 mol L^{-1} borax buffer/0.001 mol L^{-1} $Na_4P_2O_7$, pH 9.18) for 5 h at 90°C then stood at room temperature for 24 h. The resulting suspension was adjusted to 75% gel/ 25% eluent then degassed under vacuum (water pump) for *ca.* 2 h (until air bubbles were no longer released). The column (glass tubing, diameter 1.6 cm) was packed with the prepared gel, then degassed eluent was pumped through the column at 0.8 mL min^{-1} until no further settling of the gel occurred (*ca.* 6 h).

The flow rate of the eluent was calibrated on the basis of flow out of the column. Eluent was degassed before use (daily). The void volume of the column (V_0) was determined by Dextran Blue 2 000. All samples were 0.025 μm membrane filtered before being applied to the gel column. Once the use of borax/pyrophosphate eluent had been established, a single gel column was used for the majority of experiments (*ca.* 300 samples) with no change in performance (provided that care was taken to exclude air ingress).

Apparatus

Eluent was pumped through the column at a rate of 0.5 mL min^{-1} *via* a LKB 2132 Microperpex peristaltic pump. UV absorbance (280 nm) of material eluted from the column was monitored continuously with a LKB 2238 Uvicord SII detector and recorded by a LKB 2210 Potentiometric recorder. Samples (0.1 or 0.5 mL) were applied to the column by means of a Rheodyne 5020 fixed volume loop injector. Fractions were collected over 15 min intervals with a LKB Helirac fraction collector. All connections were made with Teflon tubing; the apparatus contained no metallic components.

5.2.3 Solubility of Humic Acid as a Function of pH

A weighed sample of humic acid was placed in the titration cell with an appropriate volume of electrolyte to give a concentration of *ca.* 1 mg mL^{-1}. The pH was measured with a

glass/calomel electrode pair at 25°C in a N_2 atmosphere. The pH was raised by addition of KOH and lowered by addition of HCl. Equilibria was assumed to have been attained when the pH had remained constant for at least 30 min. At selected pH values a small sample was removed (*ca.* 1 mL) and filtered (0.025 μm). The molecular size distribution for the dissolved fraction at each pH was determined by gel permeation chromatography; the area under the elution profile was taken as a measure of the amount of humic acid in solution.

5.2.4 Equilibrium Dialysis

Dialysis tubing (Spectrapor) with molecular weight cut-offs of 3 500, 12 000, and 30 000 was used. The tubing was cleaned by boiling in 2% sodium bicarbonate in 10^{-2} mol L^{-1} Na_2EDTA for 10 min, followed by rinsing with Milli-Q water, then boiling in Milli-Q water. Clean tubing was stored in Milli-Q water at 6°C.

Dialysis membranes were sealed with acid-washed Spectrum dialysis closures. Experiments were performed in acid-washed perspex cells covered with Parafilm. Solutions were stirred with a Teflon-coated magnetic follower. Disposable gloves were worn during manipulation of the dialysis tubing, and micropipettes with disposable tips were used to remove samples.

Humic acid solutions were prepared as described in Chapter 3 and 0.025 μm membrane filtered at pH 7 before use.

5.2.5 XAD Resins

Amberlite XAD-7, XAD-2, and XAD-4 resins (20 - 60 mesh) were obtained from Aldrich; XAD-8 (20 - 60 mesh) was from Sigma. The characteristics of these resins are given in Table 5.1; XAD-7 is included for comparison. Monomers and impurities were removed by hot Soxhlet extraction with AR methanol for at least 2 h. No impurities were detected in the ^1H NMR of this methanol extract. Clean resin was stored in AR methanol and washed thoroughly with triply distilled water before use.

Table 5.1: Characteristics of Amberlite XAD resins

Resin	Surface Area ($m^2\ g^{-1}$)	Pore Volume ($cm^3\ g^{-1}$)	Mean Pore Diameter (Å)
XAD-2	330	0.69	90
XAD-4	750	0.99	50
XAD-7	450	1.080	80
XAD-8	140	0.822	250

Data from Aiken et al. (1979).

5.2.6 Equilibration of Humic Acid with XAD Resins

The uptake of humic acid on several macroporous XAD resins (XAD-2,-4, and -8) was studied as a function of pH. The concentration of SHHA equilibrated with the resins (0.24 - 0.30 mg mL^{-1}) was well below the reported capacity of these adsorbents for fulvic acid (Aiken et al., 1979).

Batch experiments were performed in which an SHHA solution in 0.01 mol L^{-1} KNO_3 (0.025 μm membrane filtered) was equilibrated with clean XAD resin in a titration cell (pH *ca.* 7); dissolved oxygen was removed by purging with oxygen-free nitrogen. Both adsorption and desorption processes were studied. The solution was acidified to pH 2.5 - 3.0 by addition of HNO_3 (vapour distilled) to effect adsorption of humic acid on the resin. To desorb the humic acid the pH was raised to 11 - 13 by addition of KOH.

The resin was allowed to equilibrate with the humic acid solution for at least 1 h at each pH value; equilibrium was attained within 30 min. The concentration of humic acid in solution at each pH and its molecular size distribution was determined by UV-visible spectroscopy and gel permeation chromatography respectively. Each sample (*ca.* 1 mL) was 0.025 μm filtered before being applied to the gel column.

5.3 RESULTS

5.3.1 Solubility and Fractionation of Humic Acid as a Function of pH

The solubility of humic acid was measured as a function of pH in: KNO_3 (0.10, and 0.60 mol L^{-1}), sodium pyrophosphate, $Na_2P_4O_7$, (0.10 mol L^{-1}), and synthetic seawater.

Solubility and Molecular Size Fractionation of Humic Acid in KNO_3 and $NA_4P_2O_7$

The solubility of SHHA as a function of pH in 0.10 and 0.60 mol L^{-1} KNO_3 and in 0.10 mol L^{-1} $Na_4P_2O_7$ media is summarized in Figure 5.1 (arbitrary units).

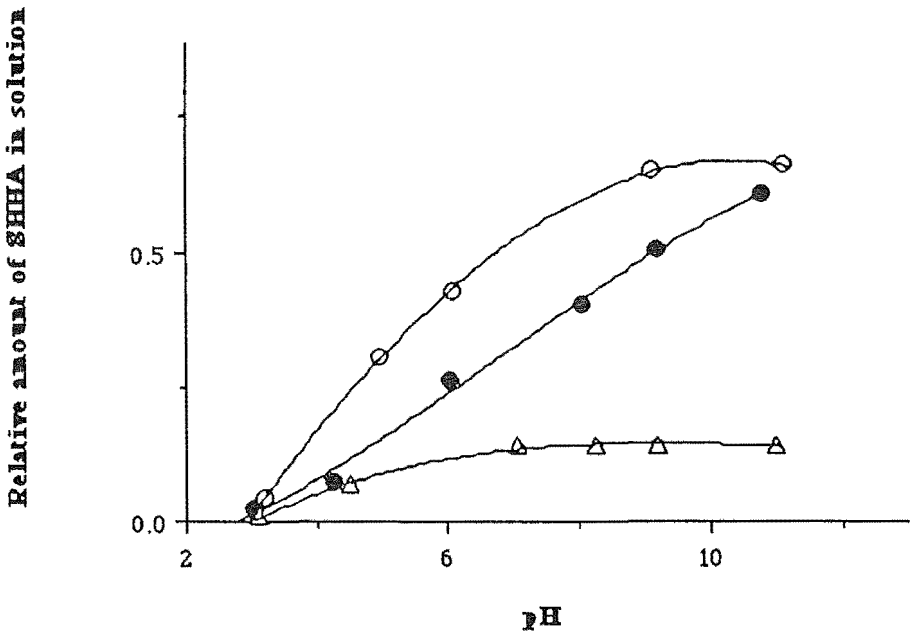


Figure 5.1: Solubility of SHHA as a Function of pH

O, 0.10 M KNO_3; ●, 0.60 M KNO_3; Δ, 0.10 M $Na_4P_2O_7$.

The corresponding molecular size fractionation is given in Figures 5.2 (0.10 mol L^{-1} KNO_3), 5.3 (0.60 mol L^{-1} KNO_3) and 5.4 (0.10 mol L^{-1} $Na_4P_2O_7$). In these, and all subsequently reported chromatograms, the elution profiles in each figure are normalized to the same absorbance scale. In all cases, the proportion and amount of larger dissolved molecules increased as the pH became more alkaline. To enable a comparison to be made between the

relative amounts of 'large' and 'small' molecules (Section 5.4.2) in solution in different media, the peak heights for these fractions were measured; Table 5.2.

Table 5.2: Ratio of Large:Small Molecules in Saturated Solution of SHHA

	KNO_3		$Na_4P_2O_7$
pH	0.10 mol L^{-1}	0.60 mol L^{-1}	0.10 mol L^{-1}
5.0	1:2.0	nd	
6.0	1:0.9	1:2.9	1:2.0
7.2	1:0.5		1:1.5
8.1	1:0.4	1:1.3	
9.1	1:0.5	1:0.7	1:1.4
11.0	1:0.7	1:1.0	1:0.8
5.8[a]	1:1.0	1:1.1	
3.1[a,b]	1:3.3	1:2.0	

[a]pH lowered to this value after exposure to alkaline conditions.

[b]solution held at this pH for 2 h.

The total amount of humic acid solubilized in 0.10 mol L^{-1} $Na_4P_2O_7$ was much less than that in 0.10 or 0.60 mol L^{-1} KNO_3 media (Figure 5.1). To investigate whether this was due to some property of the pyrophosphate, or was merely an ionic strength effect, the solubility of SHHA in 0.001 mol L^{-1} $Na_4P_2O_7$ was measured at pH 9.1. After equilibration of a 1 mg mL^{-1} SHHA solution at this composition for 4 h a spike of pyrophosphate was added to give a concentration of 0.01 mol L^{-1}; another test sample was taken after a further 4 h. In 0.001 mol L^{-1} $Na_4P_2O_7$ the amount of SHHA dissolved was similar to that in 0.60 mol L^{-1} KNO_3. The proportion of large molecules in solution increased markedly as the concentration of pyrophosphate was decreased; Figure 5.5.

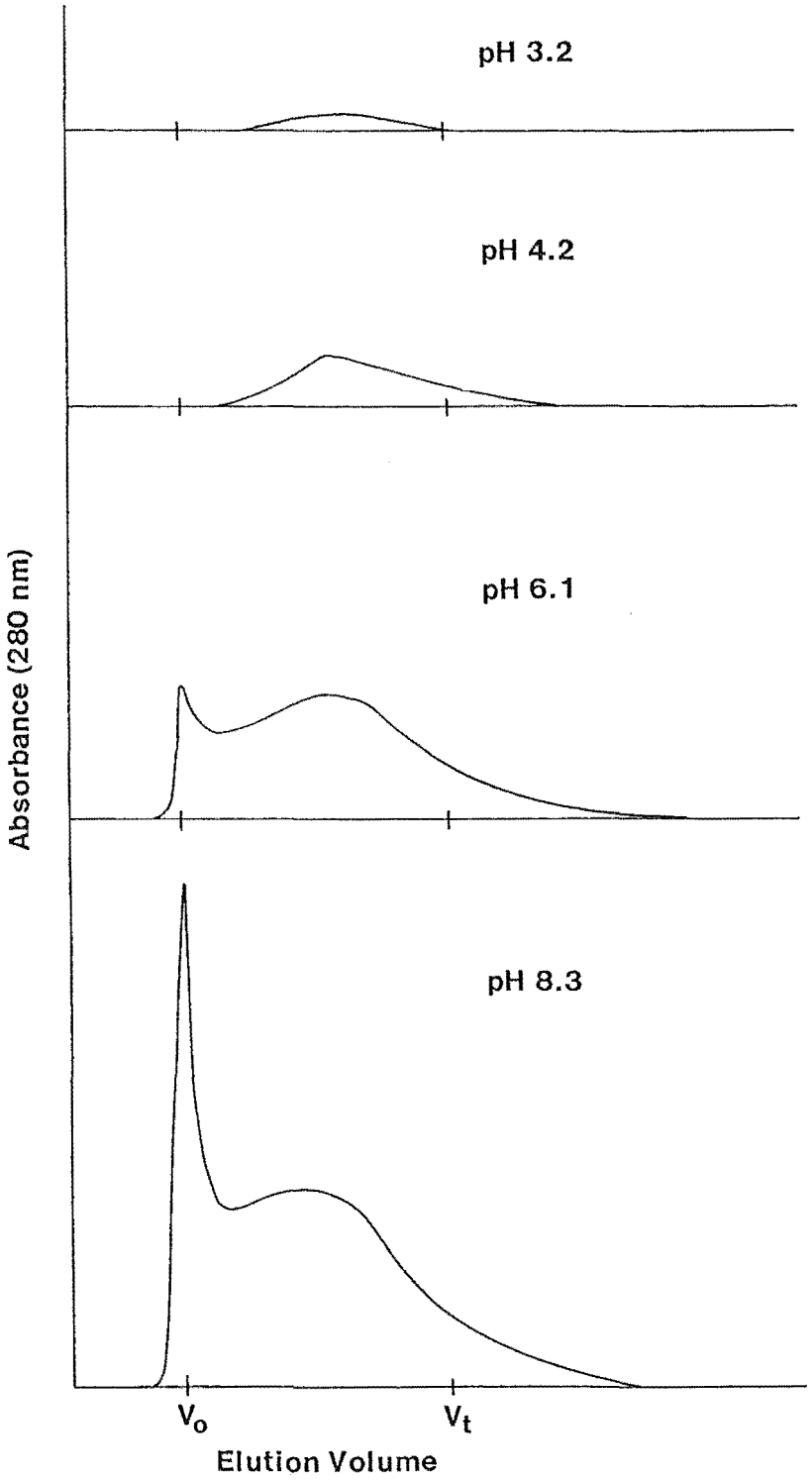


Figure 5.2: Molecular Size Fractionation of SHHA as a Function of pH; 0.10 M KNO_3

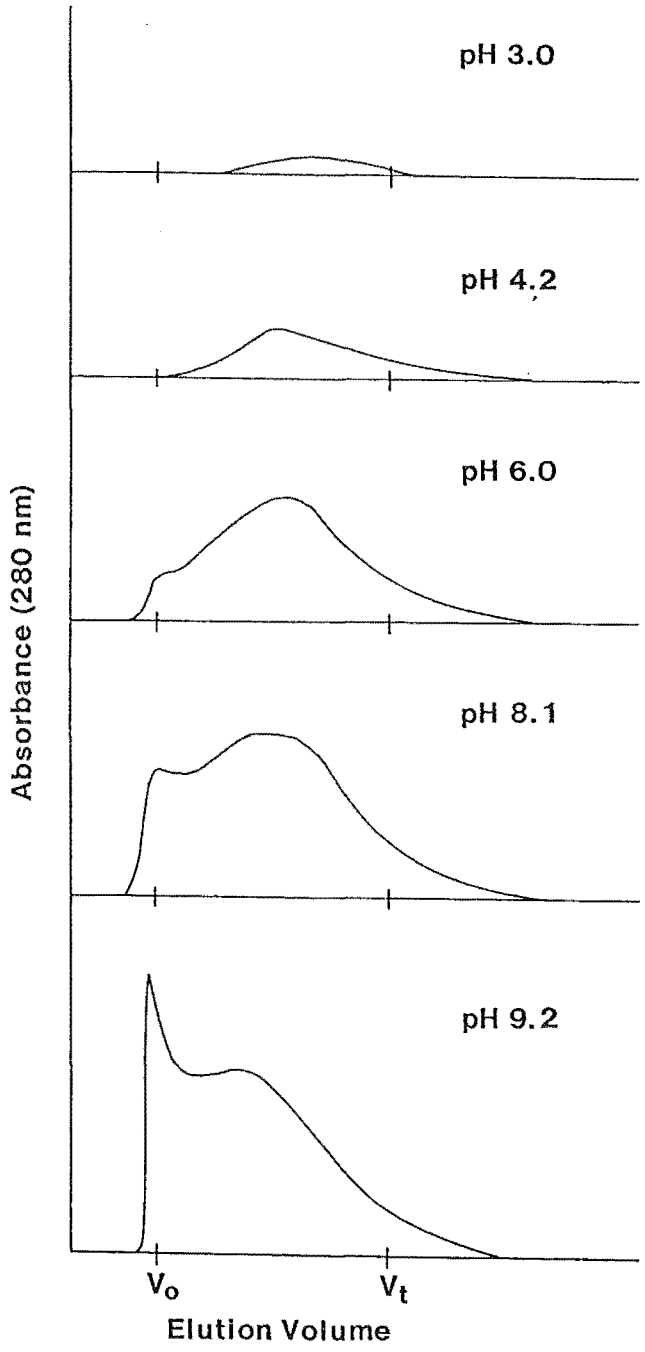


Figure 5.3: Molecular Size Fractionation of SHHA as a Function of pH; 0.60 M KNO_3

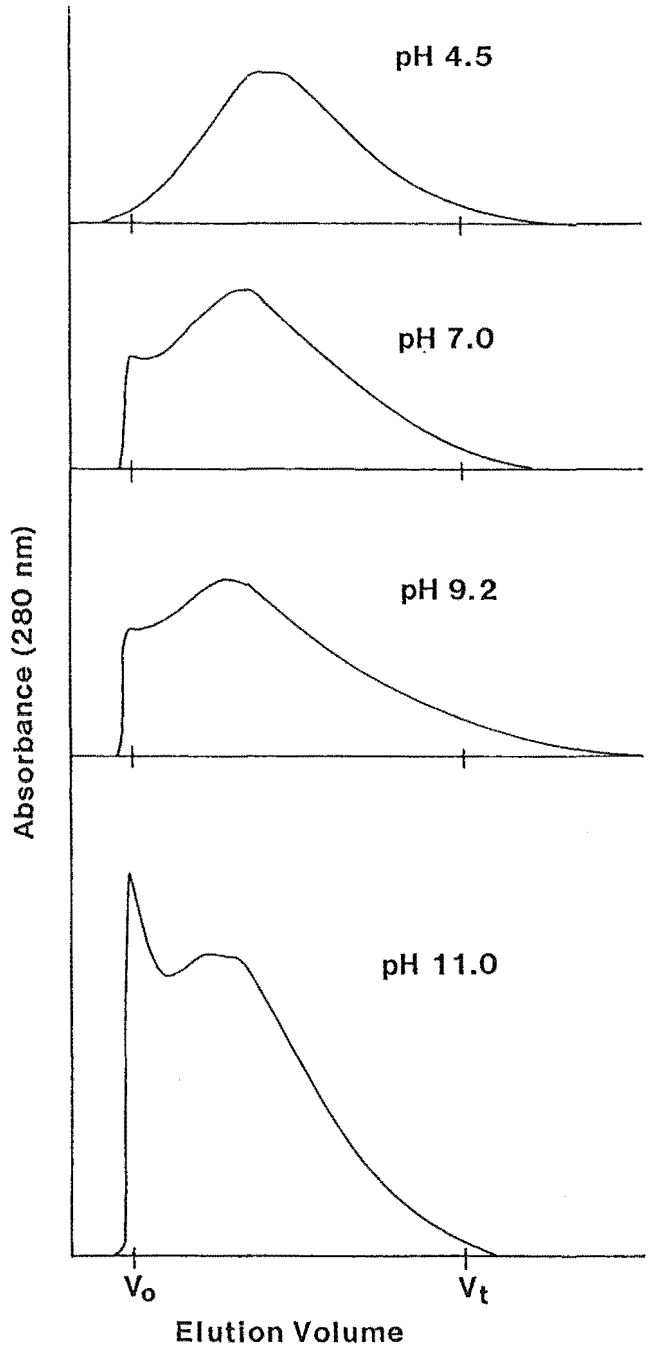


Figure 5.4: Molecular Size Fractionation of SHHA as a Function of pH; 0.10 M $Na_4P_2O_7$

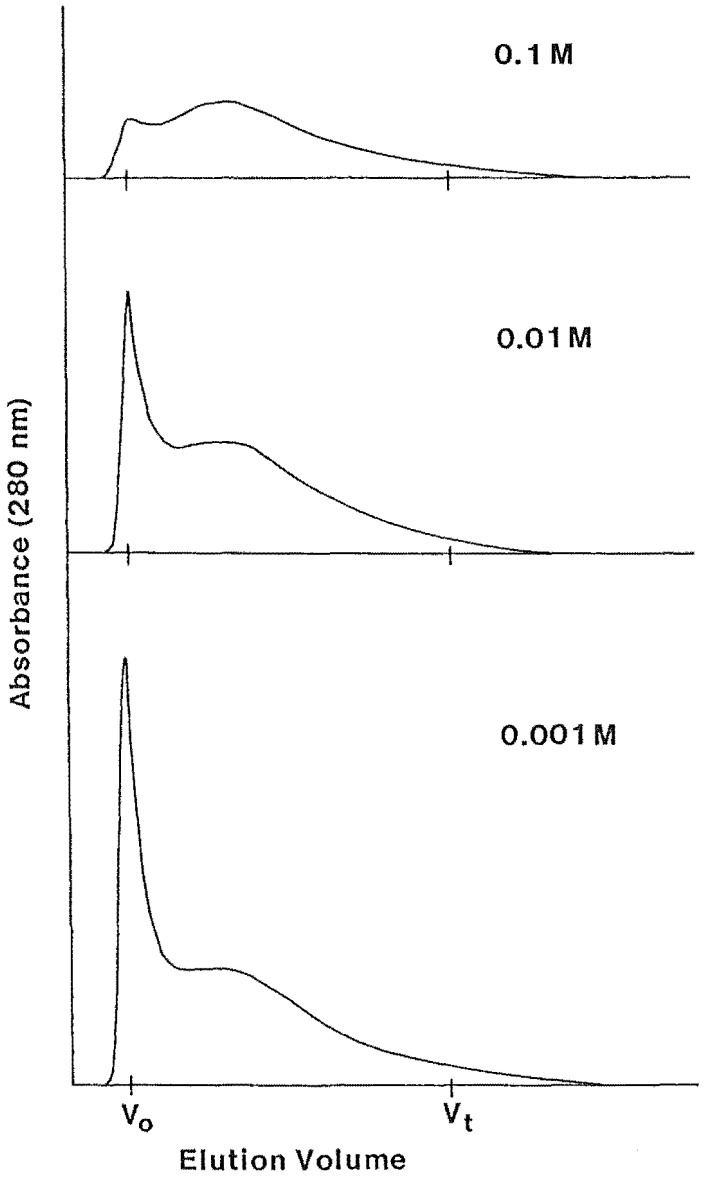


Figure 5.5: Molecular Size Distributions for SHHA in $Na_4P_2O_7$; pH 9.1

Solubility and Molecular Size Fractionation of Humic Acid in Synthetic Seawater 5

To test the effect of divalent ions on solubility, SHHA was first dissolved in synthetic seawater (pH 8.2) excluding the Ca(II) and Mg(II) content. After equilibration in this medium, Ca(II) and Mg(II) were added separately (as chlorides) with each solution being left for 2 h prior to removal of a test sample. Results are given in Table 5.3.

Table 5.3: Solubility and Fractionation of SHHA in Synthetic Seawater, pH 8.2

Solution Composition	[dissolved SHHA][a]	Large:small molecules[b]
no Ca or Mg	0.269	1:1.7
0.01 M Ca	0.219	1:1.6
0.01 M Ca + 0.027 M Mg	0.126	
0.01 M Ca + 0.053 M Mg[c]	0.054	d
0.01 M Ca + 0.053 M Mg[e]	0.108	1:2.4
0.01 M Ca + 0.053 M Mg[f]	0.115	1:3.5

[a]as determined by the area under the gel chromatography elution profile; arbitrary units.

[b]ratio of peak heights for large and small molecules.

[c]solution held at this composition for 4 h.

[d]there were no large molecules detected in solution.

[e]solution held at this composition overnight.

[f]pH 9.2.

The solubility of SHHA in synthetic seawater (sample equilibrated for 21 h in presence of 0.01 mol L^{-1} Ca(II) and 0.053 mol L^{-1} Mg(II)) was *ca.* 60% less than that in the absence of Ca(II) and Mg(II). This indicates that the presence of divalent ions does lower the solubility of humic acid. Selected molecular size distributions are given in Figure 5.6.

The solubility of SHHA in synthetic seawater was *ca.* 30% less than that in 0.60 mol L^{-1} KNO_3, although the proportion of large molecules in solution was similar.

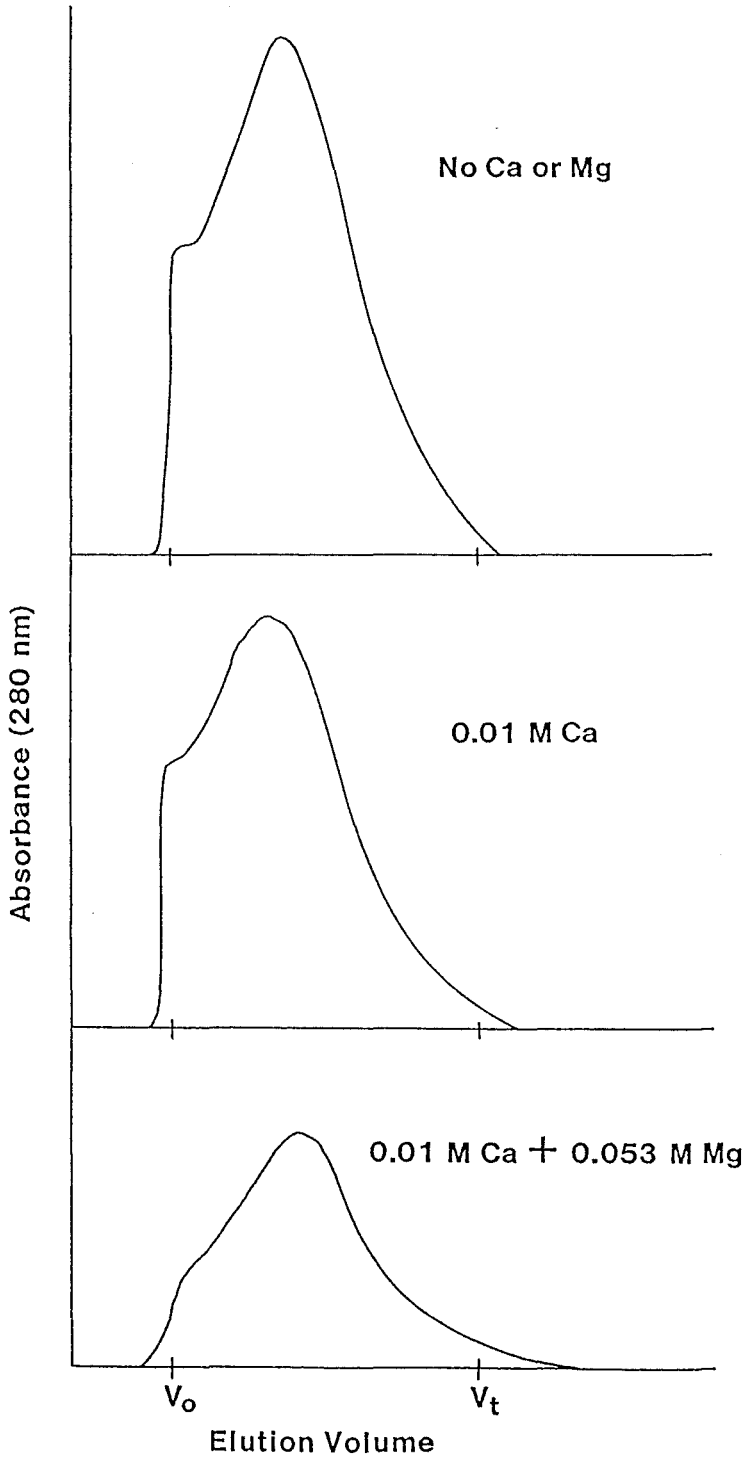


Figure 5.6: Molecular Size Fractionation of SHHA in Synthetic Seawater; pH 8.2

5.3.2 Equilibrium Dialysis of Humic Substances

Humic and fulvic acid solutions were placed inside dialysis tubing; the amount of material dialyzed and its molecular size distribution were monitored as a function of pH.

FA4

2.0 mL of FA4 solution (0.5 mg mL^{-1}; in 5 x 10^{-3} mol L^{-1} acetate buffer, pH 4.8) inside 12 000 and 30 000 MWCO dialysis tubing was dialyzed against 20.0 mL of 5 x 10^{-3} mol L^{-1} acetate buffer (pH 4.8). Solutions were stirred at this pH for 39 h, the pH was then raised to 5.5 for 24 h, then to 7.3 for 24 h. A 1.0 mL sample of dialyzate was removed at each pH.

The molecular size distribution for each of these solutions, and for the final retentate, was measured by gel permeation chromatography. In all cases a single peak was obtained but the elution volume (V_e) was different for each solution, indicating that some molecular size fractionation had occurred. For the dialyzate at pH 4.8, V_e was greater (by 4.5 mL and 4.0 mL for 12 000 and 30 000 MWCO tubing respectively) than that for an unfractionated FA4 sample; at pH 5.5 V_e was 3.5 mL and 3.0 mL greater, and at pH 7.3 V_e was 3.0 mL and 2.5 mL greater. In contrast, the elution volume for the final retentate was the same as that for an unfractionated FA4 sample, indicating that equilibration of some of the smaller molecules across the dialysis membrane did not significantly alter the molecular size distribution.

For both dialysis membranes, the concentration of fulvic acid outside the tubing at pH 7.3 was twice that at pH 4.8. To calculate the percentage of FA4 dialyzed the area under the gel chromatography elution profile was compared with that for a sample of FA4 after sample dilution by the external solution. Results are given in Table 5.4.

Table 5.4: Percentage of Fulvic Acid Dialyzed as a Function of pH

MWCO of dialysis tubing	pH 4.8	pH 5.5	pH 7.3
12 000	17	22	34
30 000	26	35	54

SHHA

1.5 mL of SHHA solution (0.2 mg mL^{-1}; in 5 x 10^{-3} mol L^{-1} acetate buffer, pH 4.8) inside dialysis tubing (3 500, 12 000, and 30 000 MWCO) was dialyzed against 20.0 mL of 5 x 10^{-3} mol L^{-1} acetate buffer (pH 4.8). Solutions were maintained at this pH for 72 h, the pH was then raised to 5.5 for 24 h, then to 7.3 for 24 h. A 1.0 mL sample of dialyzate was removed at each pH. Gel permeation chromatography was used to measure the molecular size distribution for each of these solutions (Figure 5.7), and for the final retentate.

At pH 4.8 no detectable amount of humic acid had passed through any of the dialysis membranes. At pH 5.5 no measurable amount of SHHA had passed through the 3 500 MWCO tubing, some small molecules had passed through the 12 000 MWCO tubing, and both large and small molecules were detected in solution outside the 30 000 MWCO tubing.

The total amount of material dialyzed at pH 7.3 was *ca.* 0.8 times that at pH 5.5 for the 12 000 and 30 000 MWCO membranes. The percentage of SHHA dialyzed for each dialysis membrane is given in Table 5.5.

Table 5.5: Percentage of SHHA Dialyzed as a Function of pH

MWCO of dialysis tubing	pH 5.5	pH 7.3
3 500	0	11
12 000	32	24
30 000	43	34

For the final retentate solutions, pH 7.3, the gel chromatogram peak which corresponded to the smaller molecules was displaced to lower V_e relative to unfractionated SHHA (by 3.0 mL, 2.0 mL, and 0.5 mL for 30 000, 12 000, and 3 500 MWCO respectively). This is consistent with the equilibration of a significant proportion of the smallest molecules across the dialysis membranes.

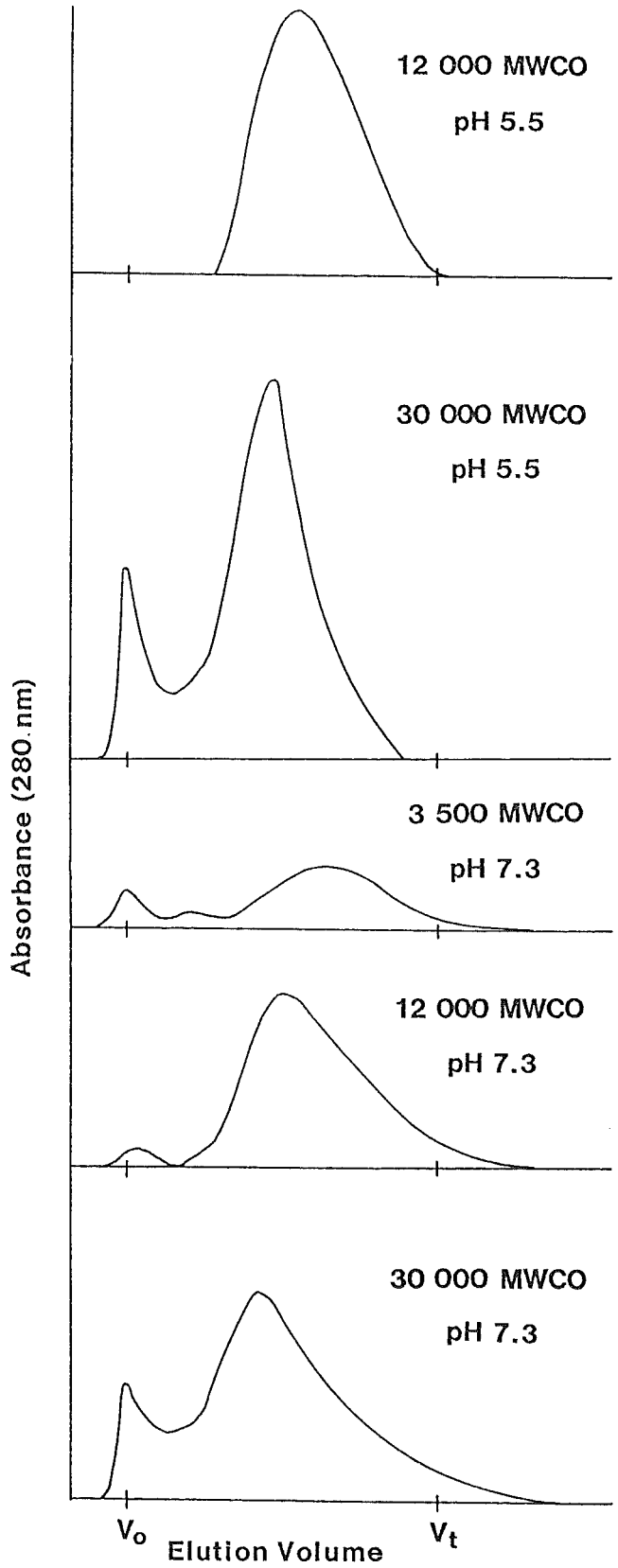


Figure 5.7: Molecular Size Distributions for Dialyzed SHHA

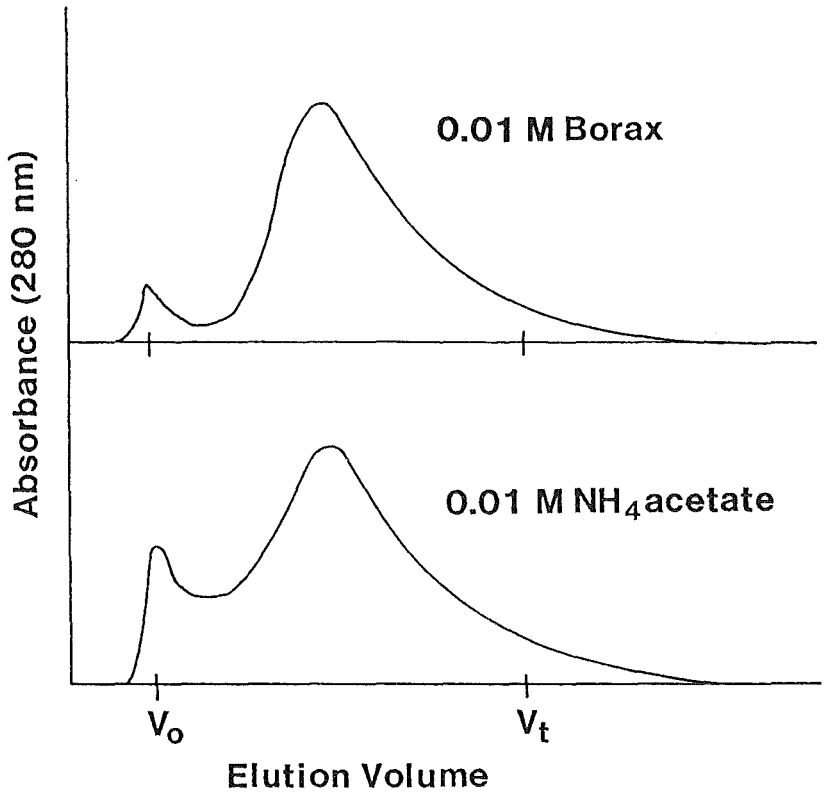


Figure 5.8: Molecular Size Distributions for Dialyzed SHHA: 30 000 MWCO Tubing

Aggregation of SHHA in Borax versus NH_3/NH_4acetate Buffer

Gel chromatography gives a higher estimate of apparent molecular weights than do other techniques (Aiken et al., 1989). To investigate whether this is a function of the borax buffer eluent (*vide supra*), 2.0 mL of SHHA solution (0.2 mg mL^{-1}, in the appropriate buffer) inside 30 000 MWCO tubing was dialyzed against 20.0 mL of 0.01 mol L^{-1} borax buffer (pH 9.18) or 0.01 mol L^{-1} NH_3/NH_4acetate buffer (pH 9.18). After stirring for 48 h, gel permeation chromatography was used to measure the molecular size distribution of the dialyzate (Figure 5.8) and of the final retentate solutions.

For both buffers a significant amount of large molecules had passed through the membrane; this effect was more pronounced for NH_3/NH_4acetate buffer. For the retentates, the peak for the small molecules was shifted to lower V_e, consistent with equilibration of small molecules across the membrane.

5.3.3 Equilibration of Humic Substances with XAD resins

Equilibration of FA4 with XAD-8

Fulvic acid is reported to be readily adsorbed on XAD-8 in acidic solution and completely desorbed at alkaline pH. Therefore, this system was studied to test the experimental procedure used in the present work.

Fulvic acid was 90% adsorbed at pH 2.0 and 100% desorbed at pH 7.0; Figure 5.9.

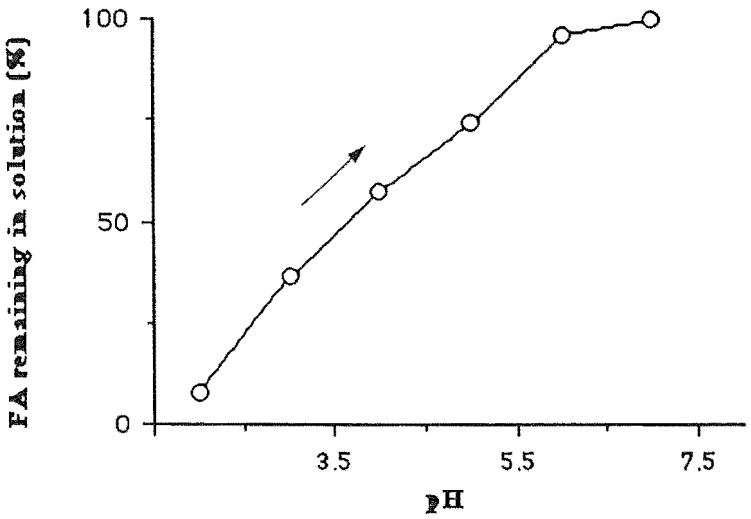


Figure 5.9: Adsorption of Fulvic Acid onto XAD-8

Equilibration of SHHA with XAD-4

The adsorption of SHHA on XAD-4 is illustrated in Figure 5.10. A neutral SHHA solution was initially equilibrated with the resin, the pH was then lowered to effect adsorption. At pH 3.0 only 24% of the SHHA was adsorbed. The molecular size distribution of these samples was not measured.

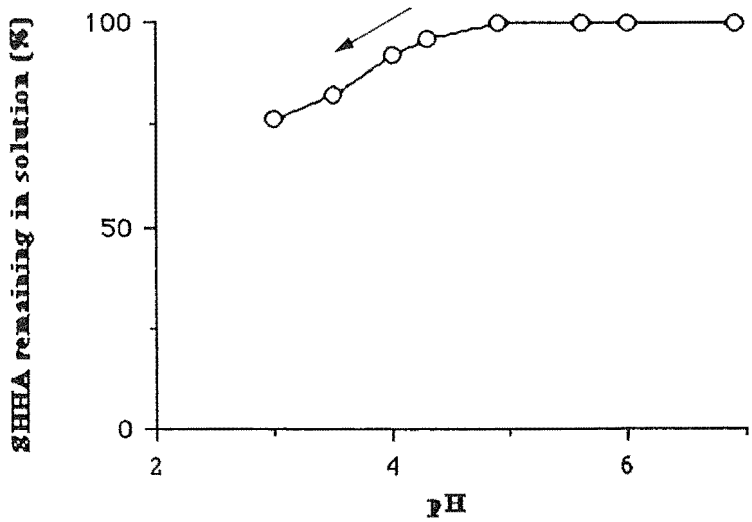


Figure 5.10: Adsorption of SHHA onto XAD-4

Equilibration of SHHA with XAD-2

Only 10% of the SHHA was adsorbed on XAD-2 following acidification to pH 3.0. This adsorbed material could not be removed from the resin on subsequent raising of the pH even after it had been stood at pH 11 overnight (data not shown).

To investigate whether preferential adsorption of a particular size fraction of SHHA had occurred on XAD-2, the molecular size distribution of the humic acid remaining in solution at each pH was measured by gel permeation chromatography. Even though the amount of humic acid adsorbed was apparently small, results presented in Figure 5.11 show preferential and substantial adsorption of small molecules at all pH values. The ratio of large:small molecules in solution was: 1:0.48 (pH 6.9); 1:0.40 (pH 6.2); 1:0.34 (pH 5.1); 1:0.31 (pH 4.2); 1:0.14 (pH 3.2). On initial equilibration of the neutral SHHA solution with XAD-2 the molecular size distribution for the soluble moieties was similar to that for unfractionated SHHA. However, following adsorption onto XAD-2 at pH 3.0 and subsequent raising of the pH to 7.0, no smaller molecules were detected in solution (not shown).

Any monomers released by the resin would not have interfered in these measurements. A 'blank' for XAD-2 equilibrated with 0.01 mol L^{-1} KNO_3 resulted in a small peak in the gel chromatogram at V_t.

After the resin containing adsorbed humic acid had been stood at pH 11 overnight, it was Soxhlet extracted with AR methanol for 6 h in an attempt to release the strongly adsorbed molecules. The molecular size distribution of this methanol extract indicated the presence of both large and small molecules. The methanol extract of 'clean' XAD-2 resin resulted in a peak in the gel chromatogram at V_t indicating the release of monomers.

Adsorption of SHHA onto XAD-2 From Pyrophosphate Solution

Pyrophosphate is a good extractant for soil humic substances (Section 5.4.5). Adsorption of fulvic acid from acidic $Na_4P_2O_7$ (0.1 mol L^{-1}) onto XAD-7 (or XAD-8) resin is a convenient way to isolate a low ash fulvic fraction (Gregor & Powell, 1986a). Therefore, the adsorption of humic acid onto XAD-2 resin in the presence of pyrophosphate was studied. Results are given in Figure 5.12. A neutral SHHA solution was initially equilibrated with the resin; on lowering the

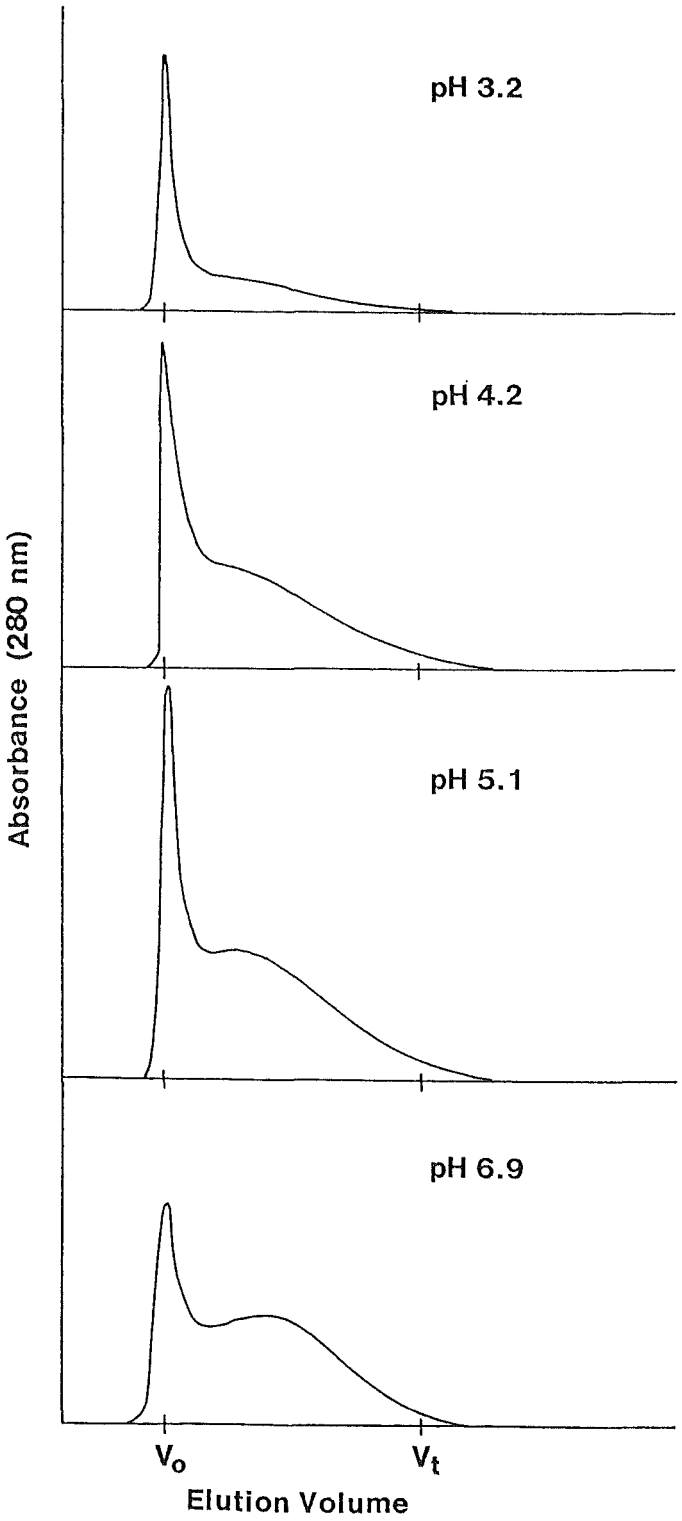


Figure 5.11: Molecular Size Distributions for SHHA Remaining in Solution in the Presence of XAD-2

pH to 2.0, 43% of the humic acid was adsorbed. Subsequent raising of the pH resulted in 35% being retained by the resin at pH 7.0; 14% was retained at pH 12.5 (not shown). (The SHHA sample was initially dissolved in KNO_3 media, followed by dilution with the appropriate volume of pyrophosphate)

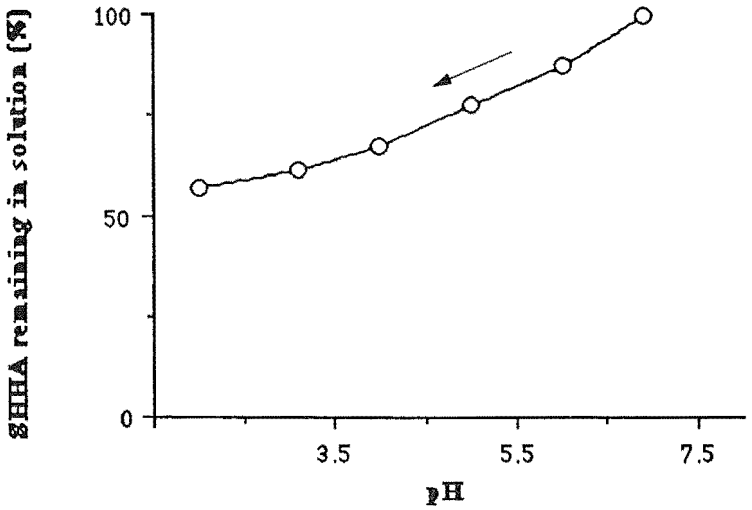


Figure 5.12: Adsorption of SHHA onto XAD-2 from 0.1 M $Na_4P_2O_7$

At the time this experiment was performed, the 'cleaning' effect of pyrophosphate on the Sephadex gel column had not been discovered; only 0.01 mol L^{-1} borax buffer was used as eluent (Section 5.4.1). Therefore, the molecular size distribution for SHHA components not adsorbed on XAD-2 from $Na_4P_2O_7$ solution could not be satisfactorily interpreted.

Equilibration of SHHA with XAD-8

The percentage of SHHA remaining in solution in the presence of XAD-8 as a function of pH is shown in Figure 5.13. A neutral SHHA solution was initially equilibrated with the resin; on lowering the pH to 2.5, 55% of the humic acid was adsorbed on the resin. Subsequent raising of the pH resulted in 10% being retained even after the resin had been stood at pH 11 overnight (not shown).

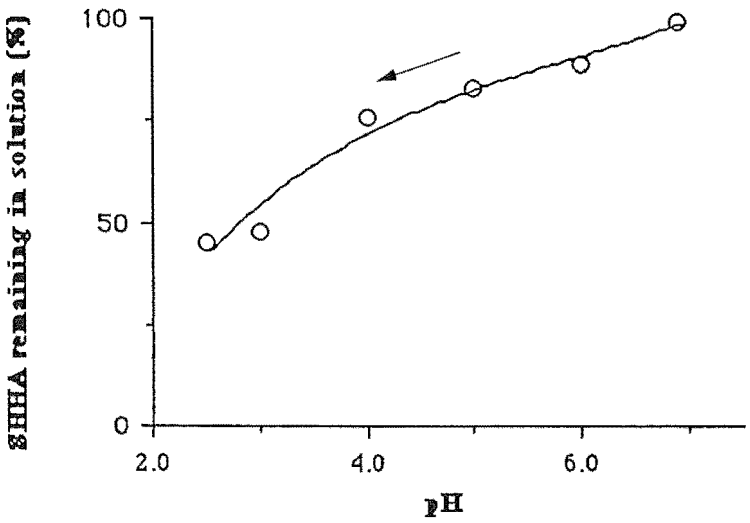


Figure 5.13: Adsorption of SHHA onto XAD-8

The molecular size distribution of the humic acid remaining in solution at each pH is given in Figure 5.14. The ratio of large:small molecules was: 1:0.43 (pH 6.9); 1:0.57 (pH 5.0); 1:0.32 (pH 4.0); 1:0.28 (pH 3.0); 1:0.24 (pH 2.5). This size fractionation of humic acid is less dramatic than was observed with XAD-2.

A sample of the XAD-8 resin at pH 2.5 and at pH 7.0 containing adsorbed SHHA was Soxhlet extracted with AR methanol. For the resin sample removed at pH 2.5 only small molecules were released by methanol extraction; both large and small molecules were released from the resin at pH 7.0.

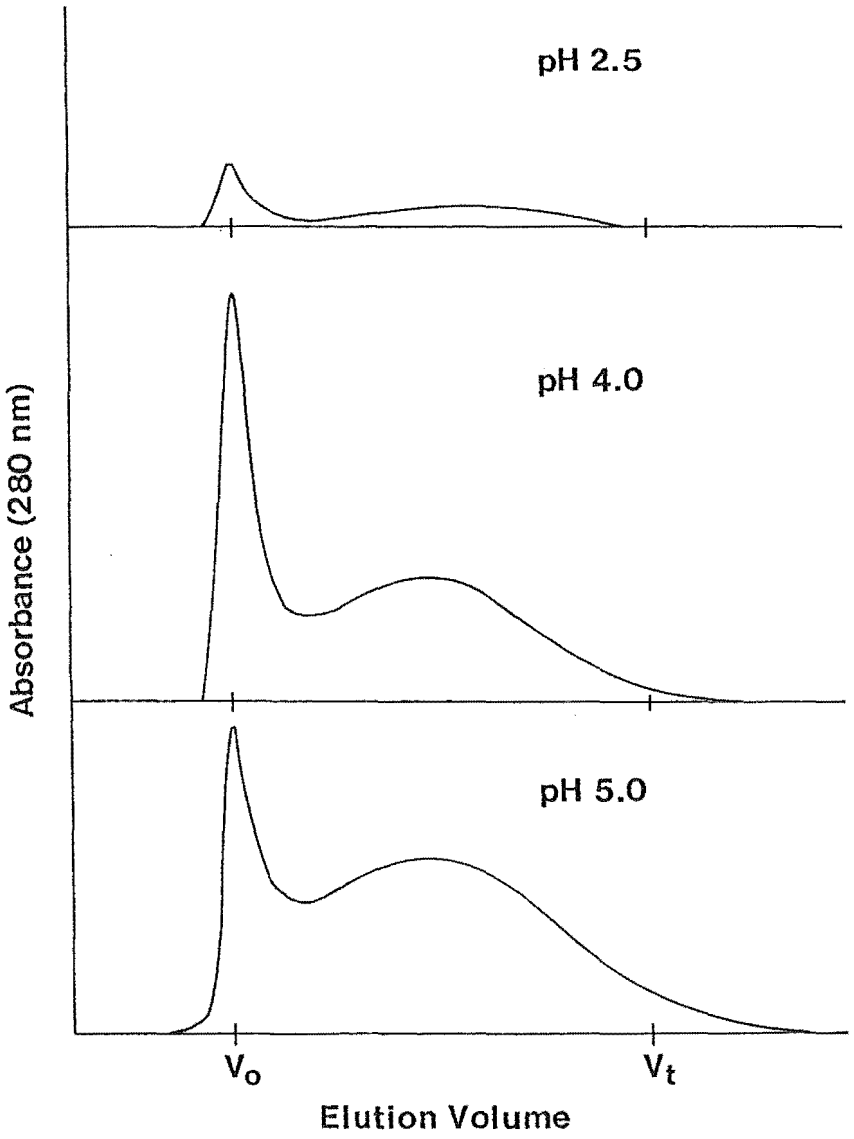


Figure 5.14: Molecular Size Distributions for SHHA Remaining in Solution in the Presence of XAD-8

5.4 DISCUSSION

5.4.1 Choice of Eluent

There has been considerable publication on the use of gel permeation chromatography to estimate the 'molecular weight' of humic substances. Adsorption phenomena are known to occur (Swift & Posner, 1971; Williams, 1972) and, indeed, have been exploited by some workers as a means to fractionate humic substances (Gjessing & Lee, 1967; Wershaw & Pinckney, 1973a; Anderson & Hepburn, 1977; Blondeau, 1986a; Haumaier et al., 1990). One aim of the present work was to minimize any adsorptive interactions between the humic substances and the Sephadex gel so that a 'true' molecular size distribution could be obtained. Under these conditions, gel chromatography can be used to *compare* the apparent molecular size distributions of different humic substance fractions. It is noted that this technique does not give an accurate estimate of the molecular weight of humic substances (Section 5.4.2).

According to Blondeau (1986a), the predominant interactions involved in gel chromatography are coulombic forces (caused by charged sites on the gel and on the solute) and adsorption forces (resulting from hydrophobic interactions). Coulombic interactions are particularly marked when the eluent is distilled water, but can be overcome by addition of an electrolyte to the eluent to suppress charges (Gelotte, 1960). The optimum concentration of electrolyte is a trade-off between two extremes. With an eluent of low ionic strength, Hall and Lee (1974) observed exclusion phenomena. This was ascribed to a mutual repulsion between carboxyl groups on the Sephadex gel and on the humic substances which would prevent the smaller humic molecules from entering the gel matrix, thus decreasing their elution volume (Hall & Lee, 1974; Mori et al., 1987). In contrast, at high concentrations of electrolyte (>1 mol L^{-1} NaCl; Kremmer & Boross, 1979) the hydration of the gel may be reduced, exposing sorption sites and thereby retarding movement of molecules through a Sephadex gel column (Janson, 1967; Sada et al., 1979; Mori et al, 1987). Further, aromatic (Gelotte, 1960; Janson, 1967), heterocyclic (Demetriou et al., 1968), phenolic (Woof & Pierce, 1967; Brook & Housley, 1969), and saturated aliphatic compounds (Hejzlar, 1987) are strongly adsorbed on Sephadex gels. Humic substances may contain all of these compounds as structural components, hence adsorption on Sephadex is

likely to occur. For high molecular weight compounds adsorption can be quite severe because multiple contacts are possible in addition to cooperative effects (Barth, 1980).

Adsorptive effects must be eliminated in order to obtain a separation based solely on molecular size differences. Such a separation is indicated if, (i) the elution volume of a substance is independent of sample concentration and flow rate, and (ii) the applied sample is completely eluted within the total column volume (V_t) (Swift & Posner, 1971; Dubin & Principi, 1989). The use of Tris and borate buffers (pH 9.2) as eluents minimized the adsorptive and the electrostatic exclusion effects in the gel chromatography of humic acid (Swift & Posner, 1971). The use of borax buffer has also been reported by Ferrari and Dell'Agnola (1963) and Söchtig (1972). Borax interacts strongly with diols (Crisponi et al., 1990) and complexes with the polysaccharide gel matrix (Kremmer & Boross, 1979) thus preventing hydrogen bonded adsorption of humic substances. Further, it is claimed that borax buffer facilitates fractionation of humic acids on Sephadex to lower apparent molecular sizes than does Tris buffer (Cameron et al., 1972a).

In the absence of adsorptive interactions, reapplication of a fraction to the gel column should yield the same elution profile as was originally obtained for that particular fraction. Such a result was reported by Söchtig (1972) for humic substances eluted with 0.02 mol L^{-1} borax buffer.

Initially, a 0.01 mol L^{-1} borax buffer eluent was used in the present work. Complete elution of humic samples within the total volume of the column was observed and there was no detectable colouration of the gel after passing 25 samples through the column. However, when samples containing sodium pyrophosphate ($Na_4P_2O_7$) were applied to the column, nonreproducible results were obtained. Large peaks near V_t were observed. After much trial and tribulation it was established that pyrophosphate was 'cleaning' the gel column. That is, even with use of 0.025 μm filtered humic substances and borax buffer eluent there was some material being adsorbed on the Sephadex gel. This adsorbed material must be a humic fraction for which pyrophosphate has a high affinity and effects solubilization, e.g. a metal-humic species. Metal complexes are known to be strongly adsorbed on Sephadex (Hirata, 1981; Adamic & Bartak, 1984) and pyrophosphate is a good complexor for metal ions, especially Al(III) and Fe(III)

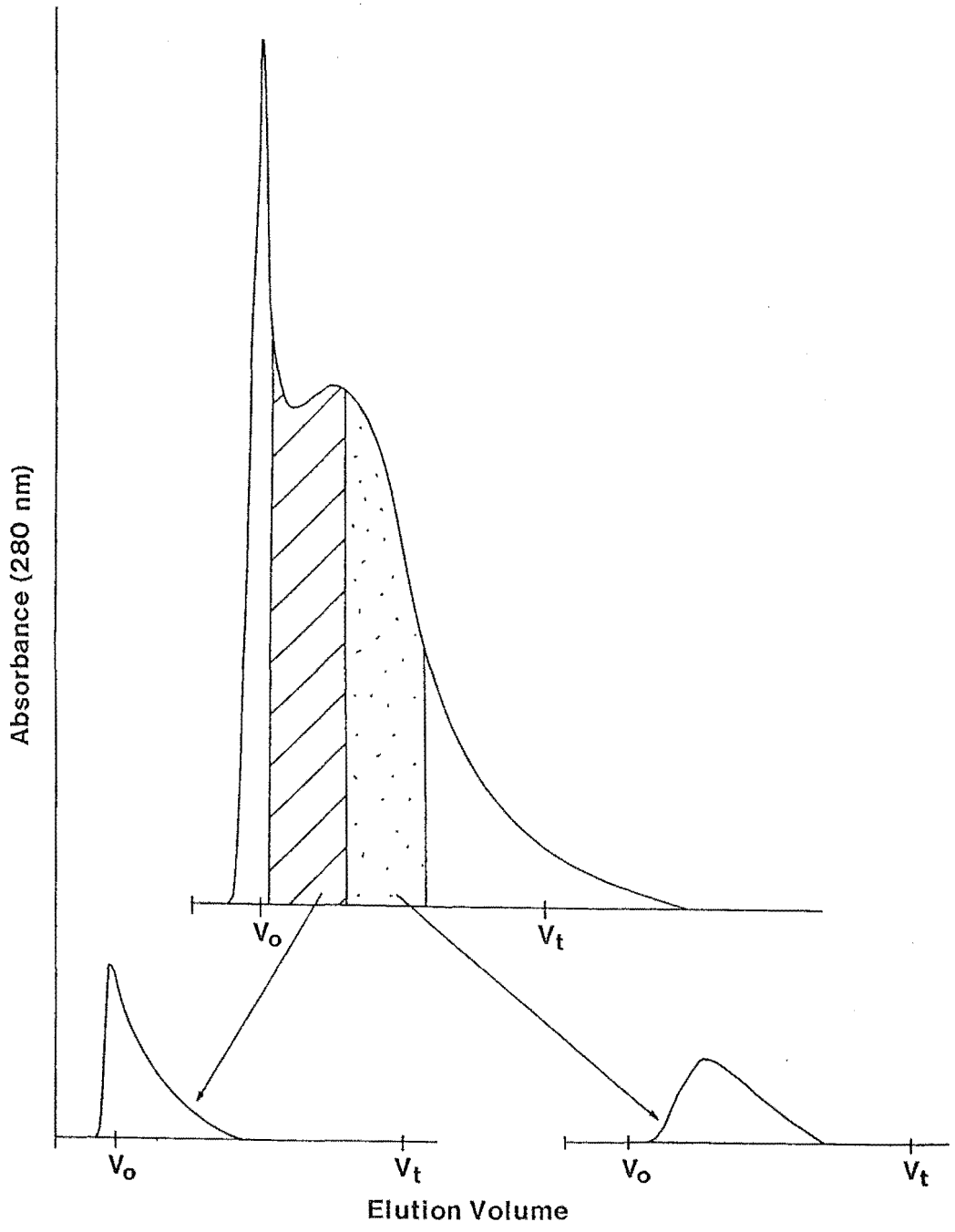


Figure 5.15: Molecular Size Distributions for Rechromatographed Humic Acid Fractions

(Bremner & Lees, 1949). Pyrophosphate also preferentially extracts organometallic complexes from soils (McKeague, 1967; Vance et al., 1985).

The use of 0.01 mol L^{-1} borax/0.001 mol L^{-1} $Na_4P_2O_7$ as the eluent prevented any build-up of humic substances on the Sephadex gel. (Pyrophosphate has no measurable UV absorbance.)

This adsorption of humic substances on Sephadex gel in borax buffer would not have been detected unless pyrophosphate had been applied to the column. The molecular size distributions obtained for humic substances in this medium were the same as those in borax buffer alone, and the pH of the eluent was not altered by the presence of pyrophosphate. No adsorptive effects were evident with the borax/pyrophosphate eluent, i.e. no sample was eluted before V_o, the entire sample was eluted within V_t, and re-application of fractions resulted in the same elution profile (Figure 5.15).

5.4.2 Calibration of the Sephadex Gel Column

Blue dextran was used to determine the void volume of the gel column, V_o. This high molecular weight (2 000 000 Dalton) coloured material passed through the column in a horizontal band, indicating that the gel was well-packed and homogeneous (Kremmer & Boross, 1979).

The separation achieved by gel permeation chromatography is dependent on the hydrodynamic size of a molecule rather than on its molecular weight (Laurent & Killander, 1964), thus separation will be governed by the shape, charge, and solvation of the solute.

The distribution coefficient, K_{av}, is used to characterize the chromatographic behaviour of solutes. $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_o, V_e, and V_t denote the void, the elution, and the total volume respectively (Söchtig, 1972). $K_{av} = 0.0$ at V_o, and 1.0 at V_t. K_{av} represents the fraction of the stationary gel volume which is available for diffusion of a given solute species. It defines solute behaviour independently of the bed dimensions and packing. A linear relationship between $\log_{10}$(molecular weight) and K_{av} has been reported for a series of globular proteins and for dextrans (Cameron et al., 1972a). However, K_{av} is correlated with molecular size only if the molecules are of similar shape and charge, i.e. a homologous series (Söchtig, 1972). This is not the case for humic substances; the heterogeneity of the sample (both in terms of shape and

chemical structure) will be important in determining the shape of the gel chromatography elution profile (Wershaw & Pinckney, 1973a). For example, although the molecular weights of two polymeric species may be identical, they could have different elution volumes if their hydrodynamic volumes are not the same.

The presence of ionic groups on a molecule can have a dramatic effect on its hydrodynamic volume. The molecular size distribution of polyelectrolytes could therefore be very sensitive to the distribution of ionizable functional groups (Barth, 1980); however, the presence of electrolyte in the eluent will reduce this effect.

Cameron et al. (1972a) attempted to calibrate a Sephadex gel column by use of "primary humic acid standards", i.e. fractionated humic samples, the molecular size of which had been calculated by combining sedimentation coefficients determined on an ultracentrifuge with diffusion coefficients (Cameron et al., 1972b). These authors anticipated that a relationship between these "primary" humic standards and "secondary" standards, such as proteins and dextrans, could be established which would allow the retention behaviour of humic acids to be predicted from the known calibration curves for the secondary standards. This would avoid the time-consuming task of preparing 'standard' humic samples for gel column calibration. However, no consistent relationship between the elution behaviour of humic acid, proteins, and dextrans was found (Cameron et al., 1972a). The primary humic acid standards used by these authors would have been polydisperse and therefore not suitable for the calibration of Sephadex (Wershaw & Aiken, 1985).

The calibration of gels with materials significantly different from those under study has been questioned (Kemp & Wong, 1974). There are no comparable reference compounds for humic substances which can be used to calibrate the Sephadex gel column in terms of molecular weights. Therefore, in the present work, gel chromatography was not used as an absolute technique, but rather as a means for *comparing* relative molecular size distributions for different humic samples (Hine & Bursill, 1984).

For humic acid solutions, two peaks in the elution profile were generally observed (depending on the pH, Section 5.4.5); one at V_0 and the other at *ca.* $(V_0 + V_t)/2$. These peaks are referred to as the "large" and "small" molecular size fractions respectively. Fulvic acid samples

eluted in a single peak at *ca.* $(V_o + V_t)/2$. To probe the probable molecular 'weights' of these fractions, a SHHA sample (0.025 μm membrane filtered; 1.0 mL) was passed through Amicon Centricon ultrafiltration membranes (10 000 and 30 000 MWCO) by centrifugation at 2 500 rpm until approximately half of the sample had gone through the filter. On application to the Sephadex gel column, the filtrate from the 30 000 MWCO filter had an elution volume 2.0 mL less than that from the 10 000 MWCO filter, indicating a larger average molecular size. Based on the molecular weight calibration of a Sephadex gel column by a series of globular proteins (supplied by the manufacturer), the molecular weight of the humic acid filtrate from both Centricon filters was *ca.* 11 000 Dalton. That is, the scale of the molecular size fractionation effected by Sephadex gel does not allow small differences to be resolved, especially for molecular weights <30 000 Dalton (even though measurable and significant changes in elution volume were observed). (Hence, the two peaks observed in the gel chromatography elution profile for humic acid may correspond to molecules of molecular weight >100 000 and *ca.* 10 000, and the peak for fulvic acid to *ca.* 10 000 Dalton.) Gel chromatography is prone to forming narrow peaks from broad molecular weight distributions because its elution volume range is so limited (Beckett et al., 1987). This highlights the use of gel permeation chromatography as only a *comparative* tool for the study of humic substances.

It is noted that there are also problems associated with fractionation of humic substances by ultrafiltration membranes; these exhibit a distribution of pore sizes. Typically, a membrane will retain 90% or more of spherical, uncharged solute molecules of the quoted MWCO (Aiken, 1984). In addition (as for gel permeation chromatography) the effective separation is dependent on the charge and molecular configuration of the particular substance being fractionated.

Further, the molecular size distributions obtained for samples applied to the gel column are controlled by the borax buffer eluent (pH 9.18). For example, the elution profile for the humic acid fraction which is soluble at pH 4.0 is measured at pH 9.18; the apparent molecular size distribution obtained may therefore be a distortion of the actual distribution in solution at lower pH (Hejzlar, 1987). Indeed, Amy et al. (1987) reported that the apparent molecular size distribution of humic substances was strongly influenced by the pH of the eluent (in borax buffer, humic molecules will be expanded due to ionization of acidic functional groups). This effect could be

caused by pH dependent adsorptive interactions between the humic molecules and the gel and/or the expanded *versus* coiled configuration of humic moieties as the pH changes (Ghosh & Schnitzer, 1980). Amy et al. (1987) emphasized that this result does not detract from the use of gel permeation chromatography as a tool for defining *relative* differences in molecular size distributions between different humic samples.

5.4.3 Quantification of 'Large' and 'Small' Molecular Size Fractions

The ratio of peak heights for the large and small molecular size fractions (defined above) was used to compare the molecular size distributions for different humic samples. The total area under the gel chromatography elution profile was used as a measure of the total amount of humic acid in solution.

The validity of these measurements is based on the assumption that the absorptivity per unit mass is the same for each of the molecular size fractions. The UV-visible absorption spectra were featureless and were very similar for all fractions. This observation has also been reported by Söchtig (1972). It has been reported that humic substance fractions of different molecular size have different "molar extinction coefficients" (Swift et al., 1970; Wang et al., 1990); a lesser effect was observed by Reid et al. (1990). However, any effect would not change the general trends observed (Goh & Reid, 1975).

The UV-visible absorption by humic substances may well be different in different media, e.g. KNO_3 *versus* $Na_4P_2O_7$. For example, it has been reported that, although an 0.5 mol L^{-1} NaOH soil extract contained almost twice as much organic matter as did a neutral 0.1 mol L^{-1} $Na_4P_2O_7$ extract of the same soil, the $Na_4P_2O_7$ extract was much darker in colour (Bremner, 1949).

In the present work, gel permeation chromatography elution profiles were monitored at 280 nm. Goh and Reid (1975) investigated the use of several wavelengths (260 nm, 465 nm, and 660 nm) for this purpose. The molecular size distributions they obtained agreed to within 10%, with that measured at 465 nm favouring a higher proportion of smaller molecular size moieties. Due to the very high UV absorptivity of humic substances, a wavelength in this spectral region is recommended for greatest sensitivity and reliability of measurements (Goh & Reid, 1975).

Söchtig (1972) monitored the elution profiles for humic substances at 254 nm, 436 nm, and 578 nm; the peak height maxima and the K_{av} values were the same at each wavelength.

5.4.4 UV Absorption by Inorganic Ion Pairs

In several media used in this work, peaks were obtained in the gel chromatograms at V_t. By applying aqueous electrolyte samples to the gel column it was established that these were caused by inorganic ions, e.g. NO_3^-, or ion pairs, e.g. $MgCl_2$, $CaCl_2$, or $Ca_2P_2O_7$. UV absorption by such species is well documented (Haddad & Heckenberg, 1983, 1984). For clarity, these peaks have not been included in the gel chromatograms reported herein.

5.4.5 Solubility and Fractionation of Humic Acid as a Function of pH

This was studied in KNO_3, $Na_4P_2O_7$, and synthetic seawater media. In summary, the solubility of humic acid increased with pH and decreased with ionic strength. By use of gel permeation chromatography it was established that predominantly low molecular size humic moieties are in solution at low pH; the proportion of soluble large molecular size components increased with pH and decreased with ionic strength. A specific chloride ion effect was also observed. These results and their implications for the understanding of the behaviour of humic substances in soils and natural waters, and for the establishment of extraction protocols are now discussed.

<u>KNO_3</u>

The effect of ionic strength on the solubility of humic acid is illustrated by the data obtained in 0.10, and 0.60 mol L^{-1} KNO_3; Figure 5.1. The relative effect was greatest at low pH, with the solubility of SHHA in 0.60 mol L^{-1} KNO_3 being 60% of that in 0.10 mol L^{-1} KNO_3 at pH 3.0, and increasing to 92% at pH 10.8.

The proportion of larger molecular size moieties in solution increased with pH and the solubility of the larger molecules at low pH was suppressed at higher ionic strength (compare elution profiles for SHHA at pH 6 in 0.10 mol L^{-1} KNO_3 (Figure 5.2) with those in 0.60 mol L^{-1}

KNO_3 (Figure 5.3)). The ratio of peak heights for large:small moieties was also greater at lower ionic strength for all pH values.

These results have important implications for the extraction of humic substances from soils and natural waters. They indicate that a pH of at least 8.0 is necessary to obtain a representative sample of humic acid in solution, and that an extractant of low ionic strength should favour solubilization of humic acid. The requirement of alkaline solutions for the extraction of humic acid is well documented (Evans, 1959; Hayes, 1985). This pH-dependent molecular size fractionation also has implications for studies on the chemistry of humic substances. For example, in titration curves of humic acid with metal ions, a different molecular size fraction may dominate solution complexing at each pH (Chapter 6).

The observed molecular size fractionation with pH is consistent with the larger humic acid molecules containing a lower density of carboxyl groups than do the smaller molecules. The ratio of large:small molecules in solution was constant above pH *ca.* 8.0. As the pH is increased, hydrogen-bonding interactions (which cause aggregation) will be minimized; this would facilitate solubilization of the larger molecules (Ritchie & Posner, 1982; Leenheer, 1985). This may also correspond to a significant contribution from phenolic groups to the acidity of the larger molecules.

After exposure to alkaline conditions, acidification of the humic acid solution indicated that re-equilibration of the molecular size fractions between the solid and aqueous phases was a slow process (although the effect of pH on the molecular size distribution of humic acid is reversible (De Haan et al., 1983)). When a solution was initially saturated with SHHA at pH 3.0, no large molecules were present in solution. After raising the pH to *ca.* 11.0, then lowering to pH 3.0 a small amount of large molecules remained in solution after 2 h. However, on standing overnight only small molecules were left in solution. Further, the amount of material in solution after being stood at pH 3.0 overnight, following exposure to alkaline conditions, was 2.4 times greater than that obtained on initial saturation of the solution at this pH in 0.10 mol L^{-1} KNO_3; for 0.60 mol L^{-1} KNO_3 the factor was 4.2. This indicates that once the larger molecules are dissolved at pH >8.0 they may exist in a meta-stable state in association with the smaller molecules. The enhanced solubility at low pH following alkaline treatment may indicate some alteration of the

humic acid, such as hydrolytic degradation of larger molecules (even though the humic acid was originally isolated with 0.1 mol L^{-1} NaOH). It is interesting to note that after exposure to alkaline conditions, lowering the pH to 5.7 resulted in the same solubility and molecular size distribution as was originally obtained.

The method used to monitor the solubility and fractionation of humic acid involved removing successive samples from a saturated solution over a range of pH values. It is possible that the removal of a sample at low pH distorted the molecular size distributions measured at higher pH. To test this possibility, the molecular size distribution for the soluble fraction of a humic acid solution saturated at pH 8.2 was compared with that for a sample saturated at the same pH but from which several aliquots had been removed at lower pH. The ratio of peak heights for the large and small molecules in the gel chromatography elution profile for each of these solutions was the same. Hence, successive removal of samples from a saturated humic acid solution at low pH would not have altered the measured molecular size distributions for samples at higher pH values.

Previous Work

Previously, the fractionation of humic substances with pH has only been studied rather crudely, i.e. at pH 1 fulvic acids are soluble, whereas humic acids precipitate. Further, earlier studies have not investigated the *soluble* humic acid fraction (i.e. that material which is not retained on a 0.025 μm filter); the entire humic substance solution including particulate and colloidal material has been studied. For example, Senesi et al. (1977) used scanning electron microscopy and electron spin resonance to probe the effect of pH on the shapes, dimensions, and extent of aggregation and dispersion of humic and fulvic acids. These authors reported that humic substances were aggregated at low pH but became more dispersed as the acidic functional groups were ionized at higher pH. In several studies on development of extraction protocols for humic substances, the molecular size of the material isolated by different techniques has been compared by gel permeation chromatography. However, detailed studies over a wide pH range have not been reported, humic samples are not filtered before application to the gel column, and frequently distilled water eluent was used (Butler & Ladd, 1969; Goh & Reid, 1975).

Effect of Triton X-100 on the Molecular Size Distribution of Humic Acid

The effect of the nonionic surfactant Triton X-100 on the molecular size distribution of the small humic acid molecules was measured. It was thought possible that a low concentration of this surfactant could enhance aggregation of humic molecules.

An eluted fraction containing small humic acid molecules was concentrated approximately 4-fold and an aliquot of Triton X-100 was added which corresponded to *ca.* 5% of the mass of the humic sample. This solution was then applied to the gel column. Triton X-100 effected no change in the apparent molecular size distribution of the small humic acid molecules (even at 25% of the mass of the humic sample).

Pyrophosphate

Aqueous alkali (0.10 - 0.50 mol L^{-1} NaOH) has been widely used as an extractant for soil humic substances, but there has also been extensive publication on the use of neutral or alkaline pyrophosphate solutions for this purpose (Bremner & Lees, 1949; Evans, 1959; Aleksandrova, 1960; Posner, 1966; Butler & Ladd, 1969; Goh & Reid, 1975; Ramunni & Palmieri, 1985; Piccolo & Mirabella, 1987; Schnitzer & Schuppli, 1989a; Piccolo et al., 1990). Acidic pyrophosphate (0.10 mol L^{-1}, pH 2.0) has been used to release fulvic acids from soils, with subsequent isolation by adsorption on XAD-7 methylmethacrylate resin (Gregor & Powell, 1986a). These authors reported that co-extraction of humic acid was minimal under these conditions.

Some authors have reported that a greater proportion of soil carbon is extracted by 0.5 mol L^{-1} NaOH than by alkaline pyrophosphate (pH 9 - 10) (e.g. Yuan, 1964). However, these differences may not be significant and will vary from soil to soil (Aleksandrova, 1960; Dormaar et al., 1970; Dormaar, 1972; Beckwith & Nayyar, 1984; Schnitzer & Schuppli, 1989a) and may depend on the particular soil horizon (Page & De Kimpe, 1989), and on the humic:fulvic ratio (Aleksandrova, 1960).

Aqueous NaOH solutions may alter the humic materials. For example, under alkaline conditions autooxidation of organic constituents may occur in contact with oxygen, amino acid and sugar polymers may be hydrolyzed, and condensation reactions between amino compounds and

aldehydes or phenolic compounds may occur (Tinsley & Salam, 1961). NaOH may also cause decarboxylation and break aromatic rings (Kallianou et al., 1987). Further, nonhumified material may be extracted (Tinsley & Salam, 1961; Ertel & Hedges, 1985; Vance et al., 1985; Schnitzer & Schuppli, 1989b); this could account for the apparent higher extraction efficiency of NaOH reported by some workers.

Therefore, it is desirable to use extractants such as pyrophosphate which are milder and more selective than is NaOH. The results presented above indicate that an alkaline pH (8 - 10) is necessary to solubilize a 'representative' humic acid fraction. Indeed, the use of pyrophosphate at pH 9 - 10 is recommended for optimum extraction efficiency (Choudhri & Stevenson, 1957; Schnitzer et al., 1958; Bascomb, 1968). A serious drawback in the use of alkaline extractants is the propensity for degradative processes to occur in the presence of oxygen. However, the O_2 uptake by $Na_4P_2O_7$ solutions (pH 9) is only 5% of that by 0.5 mol L^{-1} NaOH (pH >12) (Bremner, 1950). Any extractant for soil humic substances must be a good complexor for the metal ions which are likely to bind organic matter to soil particles, e.g. Al(III) and Fe(III); pyrophosphate fulfills this requirement (Bremner & Lees, 1949).

There has been only limited publication on the humic molecular size fractions extracted by pyrophosphate and NaOH solutions. By use of Sephadex gel chromatography (with Tris buffer as eluent), Piccolo and Mirabella (1987) reported that 0.5 mol L^{-1} NaOH extracted a larger proportion of large molecules than did neutral 0.1 mol L^{-1} $Na_4P_2O_7$ (consistent with this work). A similar result was observed by Butler and Ladd (1969); however, these authors used distilled water to elute samples applied to the gel column (i.e. adsorptive interactions would have occurred). In contrast, Ramunni and Palmieri (1985) observed similar molecular size distributions for 0.5 mol L^{-1} NaOH and neutral 0.1 mol L^{-1} $Na_4P_2O_7$ extracts.

The majority of studies on the use of $Na_4P_2O_7$ as an extractant for soil organic matter have used a concentration of 0.1 mol L^{-1}. Bremner and Lees (1949) found that this was the optimum concentration required; above 0.1 mol L^{-1} there was no further increase in the amount of organic matter extracted.

The solubility and molecular size fractionation of humic acid in 0.10, 0.01 and 0.001 mol L^{-1} $Na_4P_2O_7$ was studied in the present work. Because a previously isolated and purified humic

acid sample was used, the results may be a distortion of the processes which would occur on extracting soils with these solutions; nevertheless, some interesting effects were observed.

The solubility of SHHA in 0.10 mol L^{-1} $Na_4P_2O_7$ was significantly less than that in 0.60 mol L^{-1} KNO_3 (by a factor of *ca.* 3.5 at pH 9.18); Figure 5.1. In contrast to what was observed in KNO_3 media, the solubility of SHHA in 0.10 mol L^{-1} $Na_4P_2O_7$ reached a maximum at pH 6.5 then remained constant until pH 11.0. On acidification of the solution, slow re-equilibration processes were again observed. After exposure to alkaline conditions there was 1.2 to 1.5 times more humic acid in solution at pH 3.0 than was obtained on initial saturation of the solution at this pH.

The lower solubility of SHHA in 0.10 mol L^{-1} $Na_4P_2O_7$ than in 0.60 mol L^{-1} KNO_3 could arise from ionic strength effects. For example, the solubility of SHHA was less in 0.60 mol L^{-1} than in 0.10 mol L^{-1} KNO_3. The ionic strength of a pyrophosphate solution will be pH dependent, reaching a value of 1.0 mol L^{-1} in a fully ionized 0.10 mol L^{-1} $Na_4P_2O_7$ solution. Due to their charged, macromolecular nature, the solubility of humic acids is strongly influenced by the electrolyte concentration. At higher ionic strengths the thickness of the ionic double layer surrounding the molecules is decreased, thus allowing the molecules to approach each other more closely such that intermolecular attractive forces predominate and coagulation or precipitation ('salting-out') can occur.

To investigate this effect of ionic strength, the solubility of SHHA in 0.001 and 0.01 mol L^{-1} $Na_4P_2O_7$ was measured at pH 9.18. Gel chromatograms obtained for these samples, and for that in 0.10 mol L^{-1} $Na_4P_2O_7$, are compared in Figure 5.5.

The use of more dilute pyrophosphate solutions *increased* the amount of humic acid solubilized. The solubility of SHHA in 0.001 mol L^{-1} $Na_4P_2O_7$ at pH 9.1 was comparable to that in 0.60 mol L^{-1} KNO_3 at the same pH. This is an encouraging result because alkaline pyrophosphate solutions are more chemically mild than is NaOH.

Importantly, the proportion of large molecular size moieties in solution increased significantly as the concentration of pyrophosphate was decreased. The ratio of peak heights for large:small molecular size fractions was 1:1.4, 1:0.43, and 1:0.26 in 0.10, 0.01, and 0.001 mol L^{-1} $Na_4P_2O_7$ at pH 9.18 respectively.

When a filtered (0.025 μm) SHHA solution was spiked with $Na_4P_2O_7$ (to generate a concentration of 0.001 mol L^{-1}), there was no change in the molecular size distribution of the sample. This indicates that pyrophosphate *preferentially solubilizes* the larger molecular size humic acid moieties (rather than causing some aggregation of the dissolved molecules). Pyrophosphate has an affinity for Al(III) and Fe(III); hence, the results presented above may indicate that these metal ions are associated with the larger humic molecules. This is consistent with ISE potentiometric studies on the binding of Cu(II) to different molecular size fractions of humic acid (Chapter 6).

Synthetic Seawater

The solubility (Table 5.3) and molecular size distribution (Figure 5.6) for SHHA was measured in synthetic seawater at pH 8.2. To probe the effect of divalent ions on the solubility of SHHA, measurements were made in the absence and presence of Ca(II) and Mg(II) (as described in Section 5.3.1). Several important features were observed.

The solubility of SHHA in seawater (excluding Ca(II) and Mg(II)) was *ca.* 30% less than that in 0.60 mol L^{-1} KNO_3 but the proportion of dissolved large and small molecules was similar. No significant difference in solubility was expected because these solutions are of similar ionic strength.

The solubility and molecular size distribution of SHHA in 0.60 mol L^{-1} NaCl at pH 8.2 was the same as that in synthetic seawater media (excluding Ca(II) and Mg(II)). That is, chloride ions appear to have a significant effect on the solubility of humic acids. Because humic acid will be anionic at the pH of seawater, ion-pairing with cations will be important in determining its solubility. Chloride forms stronger ion pairs with cations than does nitrate (Perrin, 1979); this could limit the availability of cations for the solubilization of humic acid.

This effect is very important in understanding the characteristics of humic acids in seawater and needs to be considered when studies on the chemical properties of humic acid are performed in this media (Chapter 8).

In the presence of Ca(II) and Mg(II) the solubility of humic acid was further decreased (Table 5.3). The solubility decreased as the concentration of divalent ions increased; this may be

caused by the increased ionic strength of the solution and/or by the formation of insoluble Ca(II)- and Mg(II)-humate complexes (Tipping & Ohnstad, 1984). Ca(II) and Mg(II) are reported to suppress the solubility of the higher molecular weight humic substances. Increasing the pH of the seawater medium, from 8.2 to 9.2, effected a small increase in solubility (*ca.* 6%).

The behaviour of humic acid in seawater is discussed in more detail in Chapter 8.

5.4.6 Equilibrium Dialysis of Humic Substances

In all the results discussed below, it is assumed that the effective pore size of the dialysis membranes is not altered by pH or by the dialyzate medium. It is noted that adsorption of humic molecules on the membranes could alter the effective pore size by changing the nature of the membrane surface charge, as reported for ultrafiltration (Aiken, 1984).

Fulvic Acid

The maximum amount of fulvic acid dialyzed was only 54% (30 000 MWCO tubing, pH 7.3); Table 5.4. This result is surprising given that recent estimates for the molecular weight of fulvic acid are typically 1 000 - 2 000 Dalton (Thurman et al., 1982; Aiken, 1984; Aiken & Malcolm, 1987; Beckett et al., 1987; Marinsky & Reddy, 1990; Reid et al., 1990). This highlights the fact that dialysis (like gel permeation chromatography) gives a measure of the hydrodynamic *size* of the molecules, rather than their weight. Further, these techniques probably measure aggregate structures rather than individual humic molecules (Orlov et al., 1971, 1975).

Fulvic acid, by definition, is completely soluble in aqueous solution at any pH. Therefore, the observation that the amount of FA4 dialyzed increased with pH suggests that the hydrodynamic size of the fulvic moieties must be decreasing.

Similar studies on the effect of pH on the molecular size of fulvic acid molecules have reported contradictory results. Consistent with the present work, Schnitzer and co-workers (Chen & Schnitzer, 1976a,b; Schnitzer, 1977) observed a decrease in the apparent size of soil-derived fulvic molecules as the pH increased (*via* electron microscopy, UV spectroscopy, and viscometry). It was argued that at low pH, fulvic acid molecules would be aggregated due to hydrogen-bonding, van der Waals forces, interactions between π electrons, and reactions between

free radical species. These forces would become less important relative to ion-ion forces as the pH increased.

In contrast, De Haan et al., (1983) reported a decrease in the molecular weight and size of an aquatic fulvic acid with decreasing pH (*via* dialysis, Sephadex gel filtration, ultrafiltration, and UV spectroscopy). Vasconcelos et al., (1989) also reported a decrease in the molecular size of a soil-derived fulvic acid with decreased pH and with increased ionic strength.

Both the metal content and the source of the humic material may need to be considered in interpretation of data. In the studies cited above, ash-free fulvic acid was not used. As the pH is increased, complexation of metals by fulvic acid will initially become significant, resulting in an increase in the apparent size of the molecules (Truitt & Weber, 1981; Ritchie & Posner, 1982). A low ash soil-derived fulvic acid (FA4, Chapter 3) was used in the present work. It is possible that the source of the humic sample and the extraction method employed has some impact on the observed aggregation behaviour.

The concentration of fulvic acid and the ionic strength of the medium used in these studies may also be important; this aspect has been the subject of debate. De Haan et al., (1983) reported that the aggregation of fulvic acid was not affected by the concentration of the sample (65 - 325 mg L^{-1}) or by the ionic strength of the medium (0.01 - 0.10 mol L^{-1}). However, evidence for aggregation of aquatic pedogenic refractory organic matter at concentrations greater than 100 mg L^{-1} has been reported (Leppard et al., 1986). Lochmuller and Saavedra (1986) observed that the molecular size and shape of fluorescent fulvic acid components varied with solution pH, ionic strength and sample concentration (using time dependent fluorescence depolarization). Evans et al. (1989) observed a strong positive correlation between the average molecular weight of aquatic organic substances and the dissolved organic carbon concentration.

Not surprisingly, all authors are able to give a 'logical' explanation for their results and can cite other studies which 'confirm' their results! The heterogeneous nature of humic substances and the fact that their chemical structure is largely uncharacterized allows explanation of almost any result. For example, consider the effect of deprotonation of acidic functional groups as the pH is increased. Deprotonation of carboxyl groups will lead to an increase in hydrogen bonding interactions with unionized weak acid groups such as phenols, resulting in an increase in apparent

molecular size (Wershaw & Pinckney, 1977; Leenheer et al., 1989a). On the other hand, the increase in negative charge due to ionized groups disrupts molecular interactions through electrostatic repulsion, causing the apparent molecular size to decrease (Leenheer et al., 1989a). It is probable that all the factors noted above are involved in determining the aggregation behaviour of a particular humic sample (i.e. metal content, ionic strength, sample concentration, extraction method and source of the humic material). The actual configuration and distribution of functional groups on the individual humic molecules may also be important.

Humic Acid

The maximum amount of humic acid dialyzed was only 43% (30 000 MWCO, pH 5.5); Table 5.5. The trend observed was the opposite to that for fulvic acid. That is, the percentage of humic acid dialyzed *decreased* with increasing pH. This indicates that the effective molecular size of the humic acid molecules has increased at higher pH values. This could be caused by metallic impurities (SHHA contained more ash than did FA4; Chapter 3) or by some pH dependent change in the intermolecular associations between humic molecules in solution. The elution volume for the smaller molecules was shifted to smaller values (larger apparent molecular size) as the MWCO of the tubing increased (Figure 5.7).

The percentage of humic acid dialyzed at pH 5.5 was greater than that observed for fulvic acid; whereas that at pH 7.3 was less (for both 12 000 and 30 000 MWCO membranes). No measurable amount of humic acid was dialyzed at pH 4.8, indicating that the effective molecular size of dissolved humic acid moieties at this pH was greater than the pore size of the 30 000 MWCO dialysis membrane. Humic acid has been described as a complex mixture of molecular aggregates having different chemical and physical properties (Wershaw & Pinckney, 1973a,b). Different aggregation behaviour was reported by these authors for different molecular size fractions of humic acid, with each exhibiting either increased, decreased, or no change in the extent of aggregation as a function of pH. Given this, it is not surprising that different global results for unfractionated humic samples may be reported by different workers.

The appearance of large molecules in solution outside the dialysis tubing is an interesting feature of these experiments (Figure 5.7). This was observed for 30 000 MWCO tubing at pH 5.5

and for all membranes at pH 7.3. This effect was not caused by leakage through the dialysis membrane; if this had occurred, the molecular size distribution for the solution inside and outside the tubing would be the same. The effective pore size of the dialysis tubing could be pH dependent. However, especially for the 3 500 MWCO tubing, it would seem unlikely that such a large change in pore size could be effected. It is possible that humic acid exists as dynamic aggregates which are 'labile'. That is, such aggregates can dissociate, pass through the dialysis membrane, then reform on the other side. These structures have been proposed to occur in fulvic acid solutions at concentrations of 0.1 - 1.0 mg mL^{-1} (Leppard et al., 1986).

If such aggregates do exist, then their association/disocciation rate is likely to be relatively slow. For example, if a rapid dynamic equilibrium existed between monomers and aggregates then one might expect only a single peak in the gel chromatograms for humic acid. Further, when fractions were reapplied to the Sephadex gel column they eluted in their original positions (Figure 5.15). A redistribution of molecular sizes may be expected for a system comprised of aggregates in rapid equilibrium with constituent monomers. According to Wershaw and Pinckney (1973a), the elution volume for an aggregated humic acid system on Sephadex gel will be dependent on the concentration of the sample if the humic acid aggregates are in equilibrium with their constituent units. This was not observed in the present work.

Humic acids are much more polydisperse than are fulvic acids (Orlov et al., 1971; Orlov et al., 1975; Thurman et al., 1982; Beckett et al., 1987) and it is possible that a dynamic equilibrium exists between at least a small proportion of the diverse, heterogeneous molecules. Indeed, Orlov et al. (1975) stated that it is difficult to determine the molecular weights of the humic acid components because "molecular weights continually transform into micellar weights". Using flow field-flow fractionation, Beckett et al. (1987) observed that the molecular weight and polydispersity of humic acids increased in the order water < soil < peat bog < lignite coal. It was suggested that this trend represents the order of increasing tendency to form aggregates from smaller molecules (Beckett et al., 1987).

However, the presence of large molecules outside the dialysis tubing could also be explained by metal contamination. Cu(II) is prone to forming complexes which bridge two organic molecules, thus increasing the apparent molecular size of the interacting species (Maggi et

al., 1984; Orlov et al., 1990). Despite exhaustive cleaning procedures, it is very difficult to remove traces of metals from dialysis membranes (Apte et al., 1989) and problems with Cu(II) contamination were encountered in another section of this work (Chapter 8, Section B). Consistent with this, the ratio of peak heights in the gel chromatograms for large:small molecules did not change systematically with the pore size of the dialysis membrane, *viz:* 1:2.2 for 30 000 MWCO, pH 5.5; 1:1.8 for 30 000 MWCO, pH 7.3; 1:10.7 for 12 000 MWCO, pH 7.3; and 1:1.8 for 3 500 MWCO, pH 7.3. It is not obvious, however, why there were no large molecules in the solution outside the 3 500 and 12 000 MWCO membranes at pH 4.8; perhaps metal contamination was introduced during sampling of the solutions.

Such metal complexes are likely to be metal-linked polymers; simple 1:1 metal-ligand complexation would not effect such a large increase in apparent molecular size. (It is interesting to note that a similar effect was not observed for fulvic acid.) These species would have to be stable in the presence of 0.001 mol L^{-1} $Na_4P_2O_7$ (in the gel chromatography eluent). This is feasible; Slavek et al. (1982) reported that 0.1 mol L^{-1} $Na_4P_2O_7$ could recover only 90% of the Cu(II) equilibrated with a humic acid suspension.

Effect of Borax Buffer on the Aggregation Properties of Humic Acid

Gel chromatography is known to give a higher estimate for the molecular weight of humic substances than do other techniques (Aiken et al., 1989).

The apparent molecular size of humic substances as determined by gel chromatography is reported to be a function of the pH and ionic strength of the eluent (Leenheer, 1984), but these results are complicated by pH and medium dependent adsorption interactions between humic molecules and the gel matrix (Amy et al., 1987). Adsorption interactions between humic molecules and Sephadex gels is minimized by use of borax buffer eluent (Swift & Posner, 1971).

Borax is known to interact with diols (Crisponi et al., 1990) which are possible structural components of humic substances. According to Ghassemi and Christman (1968), the use of borax buffer eluent in gel permeation chromatography provides a higher estimate of the apparent molecular 'weight' of humic substances than does phosphate buffer at the same pH. To probe whether the larger apparent molecular sizes determined for humic substances by gel

chromatography are caused (at least in part) by borax induced aggregation, samples of SHHA inside 30 000 MWCO dialysis tubing were dialyzed against 0.01 mol L^{-1} borax buffer (pH 9.18) and 0.01 mol L^{-1} NH_3/NH_4acetate buffer (pH 9.18).

With both buffers a proportion of large molecules was present in the solution outside the dialysis membrane; Figure 5.8. In borax buffer the ratio of large:small molecules was 1:3.9, compared with 1:1.7 in NH_3/NH_4acetate media. This difference may be significant. In the absence of metal contamination (*vide supra*), it could indicate that humic acid aggregates are more 'labile' in NH_3/NH_4acetate media.

In NH_3/NH_4acetate buffer the amount of humic acid which passed through the dialysis membrane was 26% greater than that observed in borax buffer. This observation indicates that the effective size of humic acid molecules and/or aggregates may be increased in borax media.

5.4.7 Equilibration of Humic Substances With XAD Resins

Cleaning XAD Resins

XAD-2 and XAD-4 are nonpolar, hydrophobic, styrene-divinylbenzene copolymers; XAD-7 and XAD-8 are polar, cross-linked polymers of methylmethacrylate. As supplied, these resins contain monomers and other impurities arising from the manufacturing process which must be removed before use. A range of cleaning protocols have been reported involving Soxhlet extraction of the resins with a range of organic solvents such as methanol, acetone, hexane, and dimethyl sulphoxide (Daignault et al., 1988). The amount of impurities may vary with each batch and with the particular XAD resin used. For example, higher levels of impurities have been found with XAD-4 than with XAD-2 (Tabor & Loper, 1985); similarly, XAD-7 released more artifacts than did XAD-8 (Aiken et al., 1979). Due to the high artifact levels produced by XAD-2, XAD-4, and XAD-8, Blok et al. (1983) considered these adsorbents to be unsuitable for the isolation of trace organics from water.

In the present work XAD resins were cleaned by Soxhlet extraction with Analar methanol. ^1H NMR of the methanol extract did not detect any impurities. However, some impurities were detected by UV absorption on using the 'clean' resins in aqueous solution.

Isolation of Humic Substances

The adsorption and desorption characteristics of humic substances on XAD resins were studied to investigate the utility of these macroporous adsorbents for the isolation of humic acid from soils and natural waters under chemically mild conditions.

The use of XAD resins for the extraction of fulvic acid from soil (Gregor & Powell, 1986a) or of fulvic and humic acids from aquatic sources (Leenheer & Huffman, 1976; Aiken et al., 1979; Thurman & Malcolm, 1979,1981; Leenheer, 1981; Leenheer & Noyes, 1984) is a well established technique. Indeed, this method has been adopted by the IHSS for isolation of standard humic substances samples (Thurman & Malcolm, 1981). Fulvic acids are quantitatively adsorbed (at pH 2.0) and desorbed (at pH 7.0) from XAD-7 and XAD-8; however, release of humic acid from these resins has involved the use of strongly alkaline conditions (pH 13). More recently, questions have been raised about the reliability of this XAD resin extraction procedure (Serkiz & Perdue, 1990).

The results obtained in the present work indicate that the XAD resins studied (-2,-4 and -8) are not suitable for the isolation of a soil-derived humic acid. Humic acid was neither completely adsorbed at pH 2.5 - 3.0, nor completely desorbed even after prolonged standing at pH 11. Further, size exclusion effects were observed, with the smaller humic acid molecules being preferentially adsorbed on the resins. That is, a 'representative' humic acid sample cannot be isolated by this technique. In contrast, fulvic acid was quantitatively adsorbed and desorbed from XAD-8. These results are now discussed.

The amount of humic substances equilibrated with XAD resins in the present work was below the reported capacities of these adsorbents for fulvic acid (Aiken et al., 1979). K_D, the batch distribution coefficient, is defined as: (mg of material adsorbed by the resin per gram of resin)/(mg material in solution per mL of solution) (Aiken et al., 1979). K_D determined for FA4 on XAD-8 was 240; this contrasts with a value of 604 found by Aiken et al. (1979) at much higher solution concentrations.

Equilibration of SHHA With XAD-4

As shown in Figure 5.10, humic acid was not quantitatively adsorbed on this resin. This could be related to the polarity of the resin and/or the mean pore diameter (50 Å). It is possible that the humic acid molecules are relatively too hydrophilic to be adsorbed on the hydrophobic XAD-4 resin. In addition the resin pore size may be too small for penetration of the humic acid molecules, thus considerably lowering the effective surface area available for adsorption. However, small angle x-ray scattering has shown that the radius of gyration (a measure of molecular size) of humic acid in aqueous solution at pH 5 is 1.36 - 2.06 nm (Wershaw & Pinckney, 1977). That is, the humic molecules should be able to readily enter the resin pores. Equilibrium dialysis measurements indicated that the effective size of the humic acid molecules would decrease as the pH decreased (Section 5.4.6).

No attempt was made to elute the adsorbed humic acid. Chiavari et al. (1984) observed only a 45 - 50% recovery of commercial humic acids (Aldrich and Fluka) from XAD-4 resin on elution with 5% NaOH.

Equilibration of SHHA With XAD-2

The polarity of XAD-2 is similar to that of XAD-4 but its mean pore diameter is much larger (90 Å). However, adsorption of SHHA on XAD-2 was also minimal (in fact, it was apparently less than that observed for XAD-4). Although only *ca.* 10% of the humic acid was adsorbed on XAD-2 at pH 3.0, significant molecular size fractionation was observed, with the smaller molecules being selectively adsorbed at all pH values (Figure 5.11). Hence, this resin is also not suitable for the isolation of humic acid.

This selective adsorption could be due to size exclusion and/or to the relative polarity of the humic fractions, i.e. the large molecules may be relatively more hydrophilic than are the smaller ones. The latter seems unlikely; hydrophobicity of humic molecules is reported in increase with molecular size (Kalinowski & Blondeau, 1988).

Incomplete desorption of humic substances from XAD-2 by strongly alkaline solutions (Mantoura & Riley, 1975a; Hiraide et al., 1987) has been ascribed to charge transfer complexation (Aiken et al., 1979).

Adsorption of SHHA onto XAD-2 from Pyrophosphate Solution

The adsorption of SHHA onto XAD-2 resin from 0.10 mol L^{-1} $Na_4P_2O_7$ solution was significantly greater than that observed from 0.01 mol L^{-1} KNO_3 (Figure 5.12). This enhanced adsorption was expected, given the greater ionic strength of the pyrophosphate solution. It is noted that size exclusion effects may limit any increase in adsorption efficiency.

Mantoura and Riley (1975a) reported on 11% increase in the efficiency of adsorption of a peat-derived humic acid on XAD-2 as the ionic strength was increased from 0.0 to 0.67. Gustafson and Paleos (1971) observed a 4-fold increase in the binding capacity of XAD-2 for anthraquinonesulphonate on increasing the ionic strength from 0.0 to 0.1 mol L^{-1} (NaCl). This effect was ascribed to the concomitant decrease in dipole and coulombic repulsion of the hydrophilic groups of adjacent adsorbed molecules which increases the resin capacity (Gustafson & Paleos, 1971; Mantoura & Riley, 1975a).

Equilibration of SHHA With XAD-8

Again, humic acid was not adsorbed quantitatively by this resin. At pH 2.5, 55% of the humic acid was adsorbed (Figure 5.13); 10% could not be desorbed even after prolonged standing at pH 11.0. Size exclusion effects were also observed with this resin even though it has a mean pore diameter of 250 Å; Figure 5.14. Chiavari et al. (1984) reported an 81 - 88% recovery of commercial humic acids (Aldrich and Fluka) from XAD-8 on elution with 5% NaOH.

It is concluded that XAD-8 is also not suitable for the isolation of humic acid.

Some general features observed with all XAD resins studied and their implications for the extraction and characterization of humic substances are now discussed.

Equilibration Time

In the present work, equilibrium at each pH was attained within 30 min. Yet, Aiken et al. (1979) reported that XAD-8 had adsorbed 95% of its capacity for fulvic acid after 8 h, while XAD-4 had adsorbed only 50%. This slow uptake could be caused by the presence of particulate or colloidal material in the samples used by Aiken et al. (1979).

Size Exclusion

The present work indicates that the smaller humic acid molecules are preferentially adsorbed on XAD resins. Aiken et al. (1979) observed that the capacity of XAD-4 for molecules of 5 000 Dalton was only 1/3 of that of XAD-2; XAD-4 had no capacity for a polyacrylic acid of 90 000 Dalton. In the absence of size exclusion effects, the retention efficiency of XAD-2 and XAD-7 increased with increasing molecular weight in a homologous series (Burnham et al., 1972; Mantoura & Riley, 1975a). Fu and Symons (1989) have also reported the importance of size exclusion effects on the isolation of aquatic organic substances.

Ishiwatari et al. (1980) found that 40% of the total dissolved organic matter in a river water could be isolated by XAD-2 resin. Although the molecular size distribution for the whole water sample was not reported, that for the samples desorbed from XAD-2 indicated that the majority of the isolated material had a molecular size less than 10 000 Dalton; this could be a consequence of size exclusion. Gómez-Belinchón et al. (1988) extracted hydrocarbons and fatty acids from seawater by liquid-liquid extraction, and by adsorption on polyurethane foam and on XAD-2. The lowest proportion of high molecular weight components was isolated *via* XAD-2. These authors ascribed this observation to the association of the high molecular weight compounds with humic substances; humic-bound species would not be adsorbed on XAD-2 (Gómez-Belinchón et al., 1988). Size exclusion is also a plausible explanation for their results.

Mechanism of Uptake of Humic Substances by XAD Resins

The isolation of aquatic humic substances involves acidification of the sample to pH 2, concentration on a column of XAD resin, followed by desorption with NaOH. Gel chromatograms for humic substances desorbed from XAD-8 resin indicated the presence of a

significant amount of large molecules (Thurman & Malcolm, 1979, 1981). The present work (using a batch technique) indicated that the large molecules were excluded from XAD-2 and XAD-8 resin. Although it is likely that a proportion of large molecules are adsorbed on the resins (especially with a column technique) the possibility arises that the XAD resins are acting as a filter which traps the precipitated larger molecules. That is, for the larger molecules, precipitation/redissolution may be occurring, rather than adsorption/desorption.

It is noted that the aggregation properties of the humic molecules in a whole water sample may be different from those of the isolated soil humic acid studied in the present work.

Desorption of Humic Substances From XAD Resins

The humic acid which was adsorbed on XAD resins in acid solution was not completely desorbed at pH 11. This observation in itself is not a problem provided that the fraction of humic acid which is adsorbed and desorbed is representative of the entire sample. This was not the case; large molecules were excluded from the resin.

If a simple adsorption/desorption mechanism is in effect then the humic molecules should be completely desorbed by pH 10 - 13. Indeed, phenols and tannic acid were quantitatively eluted from XAD-8 over this pH range (MacCarthy et al., 1979). In the present work, fulvic acid was completely desorbed from XAD-8 by pH 7.0 (Figure 5.9). These results indicate that some of the humic acid moieties must interact very strongly with the XAD resin matrix and/or that some components become permanently occluded within the pores of the resin.

Specific Interactions Between Humic Acid and XAD Resins

Components with a potential to interaction strongly with XAD resins include hydrophobic moieties, aromatic species, and metal complexes.

Hydrophobic compounds are likely to be strongly retained by the nonpolar styrene-divinylbenzene resins (XAD-2 and XAD-4). For a homologous series of adsorbates the degree of adsorption from aqueous solutions by aromatic adsorbents increases as the molecular weight of the adsorbate increases, or as its aqueous solubility decreases (Gustafson et al., 1968; Gustafson & Paleos, 1971).

Aromatic compounds may interact more strongly with XAD-2 and XAD-4 than with XAD-7 or XAD-8. For example, Gustafson and Paleos (1971) reported that the capacity of XAD-4 for binding phenol was 20% greater than that of XAD-7; a two-fold difference was observed by Crook et al. (1975). Phenolic compounds cannot be completely recovered from XAD-4 on elution with organic solvents (Junk et al., 1974; Chiavari et al., 1984). Aromatic species such as pyrocatechol violet are strongly adsorbed on XAD-2 resin due to π-π dispersion forces (Brajter et al., 1988).

Metal complexes are another group of compounds which may interact strongly with XAD resins. Metal-humic complexes may be adsorbed on the XAD resin and/or the humic molecules may complex with metals already concentrated by the resin matrix. The sorption of significant amounts of metal ions on XAD-2 resin has been observed (Mackey, 1982a; Hiraide et al., 1987). Indeed, XAD-2 has been applied to the quantitative determination of Cu(II) in seawater (Sakai, 1980; Kremling et al., 1981). Wan et al. (1985) reported the use of XAD-7 for the preconcentration of metal ions from natural waters. XAD-7 and XAD-8 have capacities for metal ions which are one or two orders of magnitude greater than that for XAD-2 (Mackey, 1982b).

Irreversible Occlusion of Humic Moieties in the XAD Resin Matrix

When the XAD resins (-2, -4, and -8) were initially equilibrated with a humic acid solution at pH 7, no measurable amount of humic acid was adsorbed. Negligible uptake of humic substances by XAD resins at neutral pH has also been observed by Thurman and Field (1989). However, the present work indicated that after the solution had been acidified to effect adsorption some 10% of the humic acid could not be desorbed from XAD-2 or XAD-8 on raising the pH to 11.

In addition to the specific interactions mentioned above, it is possible that some irreversible aggregation of humic acid occurs in the pores of the resin at low pH resulting in physical trapping of the adsorbed material. The concentration of humic substances on the resin matrix is much greater than that in solution. Paleos (1969) reported that interactions between molecules bound on adjacent sites on XAD resins can occur. At concentrations greater than 1 g L^{-1} pedogenic refractory organic matter may strongly and irreversibly aggregate and dehydrate (Leppard et al.,

1986). Incomplete desorption of fulvic acid from XAD-7 resin was reported by Gregor and Powell (1986a) with some 0.04 - 0.05 mg mL^{-1} remaining on the resin at infinite dilution either at pH 6.5 or 13. This strongly retained fraction could represent irreversibly aggregated material which was physically trapped in the pores of the resin at low pH.

In an attempt to release the strongly bound humic molecules, the XAD resins were Soxhlet extracted with methanol. Application of these extracts to the Sephadex gel column revealed the presence of both large and small molecules. Changing solvents, especially changing between aqueous and organic phases, causes swelling and shrinking of XAD adsorbents which may rupture the resin beads (Daignault et al., 1988). Therefore, the large humic molecules released by methanol extraction may have been physically trapped in the resin pores.

Implications for the Structure of Humic and Fulvic Acids

The adsorption characteristics of humic acid on XAD resins may provide information on the molecular size, polarity, and aggregation tendencies of humic substances.

Aggregation Properties and Molecular Size of Humic Substances

Solubility studies (Section 5.4.5) indicated that selective precipitation of the large molecules occurs as the pH decreases, with no measurable amount of this fraction remaining in solution at pH 4 (Figures 5.2 and 5.3). However, in the presence of XAD resins there was a dramatic enhancement in the relative solubility of the large humic acid molecules (Figures 5.11 and 5.14). This effect was more pronounced for the smaller pore sized XAD-2 than for XAD-8, and was particularly marked at pH *ca.* 3. In the absence of XAD resins no large molecules were detected in solution below pH 4 (Figures 5.2, 5.3, and 5.4).

The presence of both large and small humic molecules may be a prerequisite for aggregation of humic acid. Evidence for aggregation interactions between the highest and lowest molecular weight molecules of a soil fulvic acid was reported by Wang et al. (1990).

Alternatively, the adsorption of metal ions by XAD resins (Mackey, 1982a,b; Hiraide et al., 1987) may allow the larger molecules to remain in solution to lower pH values.

The size exclusion observed with XAD-8 indicates that humic acid structures with dimensions greater than 250 Å exist in aqueous solution over the pH range studied.

Although size exclusion effects may preclude the application of XAD resins to the isolation of humic acid, these adsorbents may successfully fractionate humic acid to allow studies on the individual components. However, such a fractionation would not be clear-cut. For example, the smaller components adsorbed on XAD-2 are likely to be hydrophobic and/or aromatic in character; yet, more hydrophilic aromatic moieties, such as tannic acid, would not be concentrated on XAD-2 (Gustafson et al., 1968). Further, the large molecules remaining in solution at a given pH may in fact be aggregates of smaller components.

Polarity of Humic Moieties

The hydrophilic methylmethacrylate resins (XAD-7 and XAD-8) are reported to be more efficient for the concentration of fulvic acid than are the hydrophobic styrene-divinylbenzene based XAD-2 and XAD-4 (Gustafson et al., 1968; Leenheer & Huffman, 1976; Thurman et al., 1978; Aiken et al., 1979). The opposite has been reported for humic acids. Mantoura and Riley (1975a) observed a 95% uptake of a peat humic acid on XAD-2 at pH 2.2; for fulvic acid the uptake was 75%. In the present work the adsorption of humic acid onto XAD-8 resin was much less than that of fulvic acid. Significant adsorption of humic acid onto XAD-2 was effected in pyrophosphate media.

This indicates that the smaller humic acid molecules are more hydrophobic and/or more aromatic and/or more phenolic than are the fulvic acid moieties. Therefore, this result implies that the smaller humic acid molecules are distinctly different from fulvic acid. On a Sephadex gel column the elution volume for fulvic acid is similar to that for the small humic acid molecules. Hence, the possibility arises that the small 'humic acid' molecules are actually 'fulvic acid' which was coprecipitated with humic acid during the extraction procedure. MacCarthy et al. (1979) reported that repeated washing of humic acid precipitated at pH 1 - 2 is necessary to reduce the amount of associated fulvic acid. The humic/fulvic separation at pH 1 should be made at humic substance concentrations greater than 1 g L^{-1}. Below this concentration the separation may be incomplete due to slow precipitate formation (Malcolm, 1985). Bloomfield (1981) observed a

nearly two-fold absolute increase in the amount of fulvic acid isolated from a soil extract when part of the higher molecular weight fraction was removed by shaking the neutralized solution with clay. It was argued that this treatment reduced the amount of fulvic acid which was coprecipitated with the higher molecular weight fraction on acidification.

At least for the isolated soil humic acid studied in the present work, the characteristics of the smaller humic acid molecules are different from those of the soil fulvic acid. It is noted that because of size exclusion effects these studies do not provide any information on the relative hydrophobicity and/or aromaticity of the larger humic acid moieties. Kalinowski and Blondeau (1988) have suggested that the hydrophobicity of humic molecules increases with molecular size. According to Hayase and Tsubota (1983), the larger humic molecules have the greatest tendency to form aggregates with molecular weights greater than 10 000 Dalton. A study by Blondeau (1986b) concluded that fulvic acids are distinct chemical entities which cannot be regarded simply as a lower molecular weight fraction of humic acid.

Implications for the Extraction of Humic Substances from Soils and Natural Waters

The adsorption of humic acid on XAD resins was studied to investigate whether a method could be developed for the extraction of humic acid under chemically mild conditions. At present, all methods for the isolation of humic acid involve the use of very alkaline solutions either to leach humic acid from soil, or to desorb it from XAD resins. Because of the propensity for oxidative reactions to occur in alkaline solution, and the large proportion of nonhumic materials extracted by NaOH, it would be preferable to isolate humic acid at near-neutral pH.

The results described above established that XAD resins are not suitable for the isolation of humic acid from soil extracts. Quantitative adsorption and desorption were not obtained and significant size exclusion was observed. Although the use of an alkaline eluent increased the amount of humic acid released from these resins, such conditions offer no advantages over the traditional alkali extraction method.

An acidic solution can be used to selectively extract fulvic acid from soil (Gregor & Powell, 1986a). If soil is extracted with a neutral or alkaline reagent then the humic and fulvic fractions must be separated. This separation cannot be readily achieved by XAD resins. Although

both fulvic and humic acid are at least partially adsorbed on XAD resins in acidic solution, they are desorbed over a similar pH range. Other workers have attempted to fractionate humic substances by linear pH gradient desorption from XAD-8. MacCarthy et al. (1979) separated isolated humic substances into two fractions; carboxylic rich moieties eluted over the pH range 4 - 6, and phenolic rich moieties in the pH range 8 - 11. Ravichandran et al. (1988) attained only partial separation of humic and fulvic acids; this was ascribed to the complexity of the humic substances.

It is important to note that the humic acid used in the present work was extracted from soil by 0.1 mol L^{-1} NaOH. Nonhumic material may have been extracted by this technique (Section 5.4.5). Therefore, the question which must be addressed is "what is humic acid?". That is, the fraction of the NaOH extracted humic acid which was adsorbed and desorbed on XAD resin *may be* 'real' humic acid.

Humic substances can only be defined operationally. For both aquatic and soil-derived humic substances the separation between humic and fulvic acids is based on solubility at pH 1.0. Aquatic humic substances are further limited to that fraction of the organic carbon which is adsorbed on an XAD resin at pH 2 and desorbed at pH 13. (If a molecule contains at least one ionic functional group per 12 carbon atoms then the molecule has a solubility such that it can be isolated on XAD-8 resin and eluted by alkali (Thurman & Malcolm, 1979).)

Aquatic humic substances are reported to have different physico-chemical characteristics from those derived from soil (Hatcher et al., 1980a; Steelink & Petsom, 1987; Malcolm, 1990). In particular, aquatic humic substances are thought to be of lower molecular weight (Thurman et al., 1982; Plechanov, 1983) and to be more predominantly aliphatic (Malcolm, 1985). These observations may be an artifact of the isolation procedures. The present work has shown that the larger molecules in a soil-derived humic acid would not be concentrated on XAD resins; further, hydrophobic and/or aromatic species may be retained even at alkaline pH. According to Visser (1983), aquatic humic and fulvic acids have more characteristics in common than do those derived from soil. This observation may reflect the different isolation procedures used rather than any real differences. Indeed, Lobartini et al. (1989) compared the properties of humic substances isolated from soils and waters by XAD-8 resin and by NaOH extraction. They concluded that the method of extraction, and not the source of the humic substances, produced differences in carbon and

oxygen contents. Aquatic humic and fulvic acids extracted *via* XAD-8 had a higher aliphatic carbon content than did those extracted by NaOH. Humic acid extracted from soil by NaOH had a much greater aromatic carbon content than did that isolated by XAD-8, while that for fulvic acid was similar for both procedures (Lobartini et al., 1989).

Despite the differences which may be caused by the choice of extraction procedure, it is possible that there *are* some real differences between aquatic and soil-derived humic substances. Consider the molecular size fractionation observed as a function of pH on dissolving humic acid in various media (Figures 5.2, 5.3, and 5.4). If a significant proportion of aquatic humic acid arises from leaching of soil at pH 3 - 5, then very few large molecules will be dissolved into the water column. The smaller molecules which are preferentially solubilized in this pH range are those which are concentrated by XAD resins. Therefore, provided that size exclusion is not in effect, XAD resins may be appropriate for the concentration of humic substances from large volumes of water. Unfortunately, very alkaline solutions are still required to recover the humic acid.

Extraction of Aquatic Humic Substances

Humic and fulvic acids were extracted from a humic water (Larry's Creek, West Coast, South Island, New Zealand) using the IHSS recommended procedure (Thurman & Malcolm, 1981). The majority of the humic substances in this sample arise from leaching of surrounding Podzolized yellow brown earths.

The sample was filtered (0.45 μm) at natural pH (3.6) then acidified to pH 2.0 (HCl). UV-visible spectroscopy of the solutions at pH 3.6 and pH 2.0 (both 0.025 μm filtered) established that 60% of the coloured material had precipitated on acidification. That is, in the IHSS extraction procedure colloidal and particulate material is applied to the XAD resin. The humic substances were then concentrated by passing the solution at pH 2.0 through a column of XAD-8 resin; 13% of the coloured material was not adsorbed. Humic substances were desorbed with 0.1 mol L^{-1} AR NaOH; the humic/fulvic separation was then effected by acidifying this solution to pH 1.0 for 24 h. The precipitated humic acid was collected on a 0.025 μm membrane filter, washed with dilute HCl to remove ash, followed by Milli-Q deionized water until free of

chloride. This sample was not cation exchanged. The fulvic acid remaining in solution at pH 1.0 was readsorbed on XAD-8, washed with Milli-Q water to remove NaCl, then eluted with 0.1 mol L^{-1} NaOH. The fulvic acid was cation exchanged on Dowex 50W-8X (H form).

The molecular size distribution for the unfractionated water sample and for the isolated humic and fulvic acids was determined by gel permeation chromatography (Figure 5.16). The humic acid did contain a significant amount of large molecules, whereas none were detected in the whole water sample. It is possible that the large molecules are not present in sufficient concentration to be detected in the original sample; the isolated humic acid was concentrated *ca.* 500-fold. The apparent molecular size of the smaller humic acid components was greater than that for the unfractionated sample; that for fulvic acid was less. The apparent molecular size for the whole water sample was the same at pH 3.6 and 2.0.

The aquatic humic acid had a lower proportion of large molecules than did soil humic acid (it was also more readily soluble). This observation could be a result of the origin of the aquatic humic acid. If it arises from leaching of soil at pH *ca.* 4 then not many large molecules are expected to be solubilized.

In contrast to the experiments with soil-derived humic acid, no visible coloured material remained on the XAD-8 resin at pH 12 following concentration of aquatic humic substances. This may indicate that the components of soil humic acid which are strongly adsorbed by XAD-8 are not present in aquatic humic acid and/or are an artifact of the NaOH extraction process.

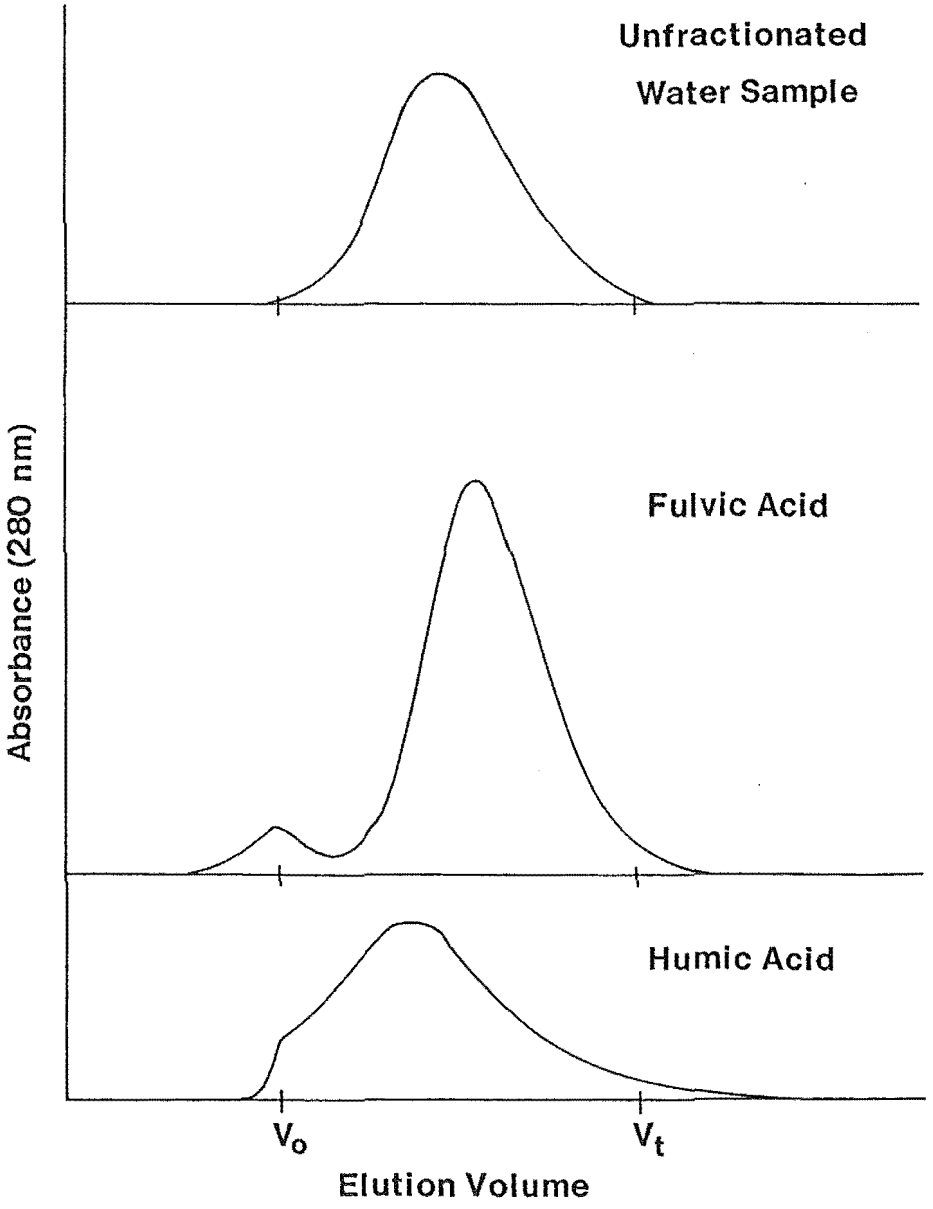


Figure 5.16: Molecular Size Distributions for Humic Substances From River Water

Conclusions

XAD resins do not provide any advantages over the conventional NaOH extraction procedure for the isolation of humic acid from soil extracts; they may actually be disadvantageous. It is not possible to isolate a 'representative' soil humic acid sample *via* XAD resins at near-neutral pH, or at least not a fraction which is similar to that extracted by NaOH.

Due to the complexity and heterogeneity of humic substances, one operational definition is probably no worse than any other, and methods involving the more chemically mild conditions should be favoured. The results obtained in the present work indicate that 0.001 mol L^{-1} $Na_4P_2O_7$ at pH 9 would be a good extractant for soil humic acid.

Recent studies have reported the use of dipolar aprotic solvents for the isolation of humic substances under more chemically mild conditions than that involved in extraction with NaOH or $Na_4P_2O_7$ (Senesi et al., 1983; Law et al., 1984; Piccolo & Mirabella, 1987; Piccolo et al., 1989). These solvents extract humic substances by disrupting intermolecular hydrogen bonds and yield samples with a low inorganic ash content. However, the extraction efficiency of these reagents was less than half that of NaOH or $Na_4P_2O_7$ and only a small proportion of the larger molecules was isolated (Piccolo, 1988).

Whatever extractant is used, some method is needed to separate the humic and fulvic fractions. As noted above, XAD resins are not suitable for this purpose and coprecipitation can occur when the separation is effected at pH 1.0. Recently, liquid-liquid partitioning of a soil extract at pH 1.0 with methyl isobutyl ketone has been reported to efficiently separate humic and fulvic acids (Thorn et al., 1987a; Rice & MacCarthy, 1989a,b). It is possible that this technique could be applied to more neutral soil extracts.

By whatever means humic substances are isolated it is important that the mechanisms of the extraction procedure are understood to allow studies on the isolated samples to be interpreted in some meaningful way. According to Piccolo (1988), dipolar aprotic solvents extract the fraction of humic substances which are not strongly linked with silicate minerals, have not undergone extensive condensation reactions, and have an increased reactivity due to their high content of acidic functional groups. In contrast,

pyrophosphate extracts high molecular weight material which was strongly bound to silicates. Humic substances with a low inorganic ash content are obtained by both methods; this is essential for studies on the isolated samples (Malcolm, 1976). For this reason, and for the propensity for degradative reactions to occur in highly alkaline solutions, the use of NaOH for the extraction of humic substances cannot be recommended.

CHAPTER 6

ION SELECTIVE ELECTRODE POTENTIOMETRIC STUDIES ON THE COMPLEXATION OF Cu(II) BY HUMIC SUBSTANCES

6.1 INTRODUCTION

Models for the calculation of metal complexation by humic substances are complex, and most involve calculation of conditional stability constants by least squares fit at one pH (e.g. Stevenson, 1977; Turner et al., 1986). Interpretation of data over a pH range is difficult. Lamy et al. (1988) concluded that it is not possible to distinguish specific Cu(II) complexation sites in humic substances, and that a mixture of different complexes with different stability constants is probably formed. Perdue (1989) has outlined the problems associated with attempts to quantitatively describe complexation of metals by humic substances. No models can quantitatively describe the effects of pH and ionic strength on the extent of complexation of a single metal ion, and multimetal binding cannot be modelled at all.

It is not even certain whether both 1:1 and 1:2 metal-to-ligand complexes are formed. The assumption that both species are formed may improve the least squares fit for the humic substance data (Buffle et al., 1977), but other models postulating multiple sites or site interactions and only 1:1 stoichiometry fit the data equally well (Cabaniss et al., 1984). It is also noted that the protonation equilibria of humic substances cannot be described quantitatively.

Metal coordination need not involve those donor groups in humic substances which deprotonate at lowest pH (as assumed by Young and Bache (1985)), nor those which are numerically dominant (as assumed by Murray and Linder (1983)). Further, the presence of different functional groups in humic substances with different protonation constants means that the coordination mode will vary with pH, ionic strength, and metal-to-ligand ratio (Cheam, 1973; Perdue, 1989). This arises from preferential complexation by stronger ligands at low metal-to-ligand ratios. Erroneously, the variation in the conditional stability constant with metal-to-ligand ratio has been cited as evidence for

two types of binding sites in humic substances (Guy & Chakrabarti, 1976; Saar & Weber, 1982; Varney et al., 1984; Coale & Bruland, 1988; Midorikawa et al., 1990).

In addition to 'simple' complexation, other reactions may occur. For example, formation of colloidal precipitates, aggregation and adsorption onto other colloidal matter, and formation of mixed ligand complexes have been proposed for the more water soluble fulvic acid fraction (Buffle et al., 1977; Rainville & Weber, 1982; Midorikawa et al., 1990). Indeed, using Rayleigh light scattering Gamble et al. (1985) distinguished between intra and intermolecular Cu(II) complexation by fulvic acid. Development of mathematical models to describe this behaviour is difficult, although Teasdale (1987) has recently reported a theoretical description of copper-induced aggregation of humic substances.

Another factor which complicates analysis of metal complexation by humic substances is that results are method dependent (Neubecker & Allen, 1983). It is important to be aware (as much as it is possible) of the specific species detected by a particular technique, and of any interfences from the humic substances themselves. For example, Cabaniss and Shuman (1986) studied Cu(II) complexation by humic substances by ISE potentiometry (to determine free metal) and by fluorescence quenching (to determine unbound ligands). These techniques gave similar results at low levels of Cu(II) complexation but became disparate as the Cu(II) concentration increased. Problems associated with comparison of data from these techniques have been discussed (Cabaniss & Shuman, 1988c; Ryan et al., 1990).

Techniques used to probe complexation of metal ions by humic substances include: pH potentiometric titration (Marinsky et al., 1982a; Buffle et al., 1990a); gel permeation chromatography (Mantoura & Riley, 1975b; Hirata, 1981); fluorescence quenching (Cabaniss & Shuman, 1986); ISE potentiometry (Buffle et al., 1980; Cabaniss & Shuman, 1988a), ASV (Kyle, 1987); fixed potential amperometry (Hering et al., 1987); chemiluminescence (Huizenga & Patterson, 1988); metal exchange with Co(III) (Hanck & Dillard, 1977); Mössbauer spectroscopy (Goodman & Cheshire, 1979); dialysis titration (Truitt & Weber, 1981; Rainville & Weber, 1982); NMR (Gamble et al., 1976); and ultrafiltration (Buffle & Staub, 1984). A factor to be considered in selecting a method is the error associated with data determined by a particular technique (Fish & Morel, 1985a,b).

The methods used to study metal-humic coordination, and their limitations, have been comprehensively reviewed (Hart, 1981; Neubecker & Allen, 1983; Jardim & Allen, 1984; Tuschall & Brezonik, 1983a, 1984; Lund, 1986; Perdue, 1989; Shuman et al., in press)

6.1.1 Scope of This Work

Many of the attempts to model metal binding properties of humic substances have not recognized the inherent complexity of these heterogeneous, macromolecular compounds. For humic substances, neither the free ligand concentration, the nature of the binding sites, nor their respective protonation constants are known. The author therefore considers quantitative analysis of metal-humic equilibria to be inappropriate. An approach is favoured in which experimental complexation curves are compared directly with those measured or calculated for finite mixtures of model ligands (Gregor, Powell & Town, 1989a,b). This allows for variation in coordination mode with change in pH. No attempt was made to calculate conditional stability constants for Cu(II)-humic substance complexes.

Ion selective electrode potentiometry, although limited by low sensitivity, has fewer problems associated with data interpretation than does ASV (Chapter 7).

6.2 EXPERIMENTAL

6.2.1 Standard Solutions

Cu(II)

Preparation and standardization of the stock Cu(II) solution is described in Chapter 3. Standard Cu(II) solutions (10^{-2} - 10^{-6} mol L^{-1}) were prepared by accurate dilution in 0.10 mol L^{-1} KNO_3 ($p[H^+]$ *ca.* 4).

HNO_3

Stock HNO_3 solutions (*ca.* 1 mol L^{-1}) were prepared by dilution of concentrated HNO_3 (BDH, Analar) with Milli-Q water. These solutions were standardized by titration against weighed amounts of Tris (Fluka, puriss p.a.)

KNO_3

KNO_3 was stored in a desiccator over anhydrous $CaCl_2$ for a least 1 month before use. A stock solution of KNO_3 (1.00 mol L^{-1}) was prepared by dissolution of the appropriate weight of KNO_3 (Riedel-de Haën, für Analyse) in Milli-Q water.

6.2.2 Copper(II) Titrations

The general titration technique, and the humic substance samples are described in Chapter 3. At the end of each titration the solution was back-titrated with standard HNO_3 (to pH 2.5 - 2.8); the ISE was then calibrated *in situ* by addition of aliquots of standard Cu(II).

Electrodes

Calomel electrodes (Radiometer K401) were encased in glass jackets containing 0.10 mol L^{-1} KNO_3 (vycor junction) to minimize chloride interference. Each electrode pair was connected to a Radiometer PHM64 pH meter.

pH was measured with a glass (Beckman)/calomel electrode pair; calibration was against NBS buffers as described in Chapter 2.

The concentration of free Cu(II) was measured with a Radiometer Ruzicka Selectrode/calomel electrode pair. The ISE was made sensitive to Cu(II) by application of a layer of electroactive Cu(II) Selectrode powder (S42015), followed by conditioning in 0.1 mol L^{-1} EDTA overnight. When not in use the ISE was stored in Milli-Q water (storage in EDTA is not advantageous (Avdeef et al., 1983)). Experiments were performed under constant light conditions because a Cu(II)-ISE is photosensitive (Cheam, 1973).

Burettes

Gilmont micrometer syringes fitted with 2.5 mL glass burette tips were used to dispense alkali, acid, and metal titrants.

Complexation Capacity Curves

Cu(II) binding as a function of metal:ligand ratio (the complexation capacity curve) was measured by incremental addition of standard Cu(II) solutions to humic substance solutions (0.017 mg mL^{-1}) at pH 5.0, 6.3, and 7.0 (I = 0.10 mol L^{-1} KNO_3) to generate total Cu(II) concentrations in the range 0.0 to 1.5 x 10^{-4} mol L^{-1}. At each datum point pCu was determined and the total free metal concentration was calculated. (Free metal = $[Cu(II)] + [Cu(OH)^{+}]$ (log β = -7.71) + $[Cu(OH)_2^{2+}]$ (log β = -10.99) (Sylva & Davidson, 1979).) pH was maintained constant by addition of standard KOH to neutralize protons released in the coordination reaction and added *via* the acidic Cu(II) solution. From an inflexion in the plot of free metal *versus* total added metal, followed by a linear portion with slope $\approx$ 1.0, it was inferred that the complexation capacity had been reached. The complexation capacity was obtained by extrapolation of the linear portion of the curve to the x-axis.

Metal Binding Curves

Metal binding as a function of pH was determined by titrating an acidic Cu(II)-humic substance solution (I = 0.10 mol L^{-1} KNO_3) with standard KOH in the pH range 2.5 - 7.5. The mass of humic substance used was such as to give a carboxyl group concentration of 1.8 x 10^{-4} mol L^{-1} (unless otherwise stated). Thus, for a 1:4.5 metal-to-ligand (COOH) ratio the concentration of Cu(II) was 4.0 x 10^{-5} mol L^{-1}, while for a 1:20 ratio it was 9.0 x 10^{-6} mol L^{-1}. pCu was determined at each datum point and the total free metal was calculated. Data were plotted as percentage free Cu(II) *versus* pH.

Simulation of Binding Curves

Metal binding as a function of pH, and of metal:ligand ratio at fixed pH, was calculated for model ligands using FORTRAN computer programs. These programs calculated the percentage of each species present at each pH, or each total Cu(II) concentration, from the known protonation and metal complexation stability constants. Stability constants measured at 25°C and I = 0.1 mol L^{-1} were chosen whenever possible (Perrin, 1979). The calculated "free Cu(II) concentration" included hydrolysis products (*vide supra*). Ligands for which disparate equilibrium models have been published, e.g. citrate and tartrate, were measured experimentally; the constants for the model which most closely represented the experimental data were used in subsequent calculations. Thus the equilibrium Cu(II) binding model for citrate proposed by Ramamoorthy et al. (1972) provided a better fit to the experimental data than that of Field et al. (1974); while for tartrate the model of Bottari et al. (1969) was superior to that of Rajan and Martell (1967).

6.3 RESULTS

6.3.1 Copper(II) Complexation Capacity Curves

Typical complexation capacity curves for humic acid at pH 5.0 and 7.0 are shown in Figure 6.1a; for clarification, data at the lower total Cu(II) concentrations are shown in Figure 6.1b. Similar curves were obtained for fulvic acid. At least two separate titrations were performed for each humic substance sample at each pH.

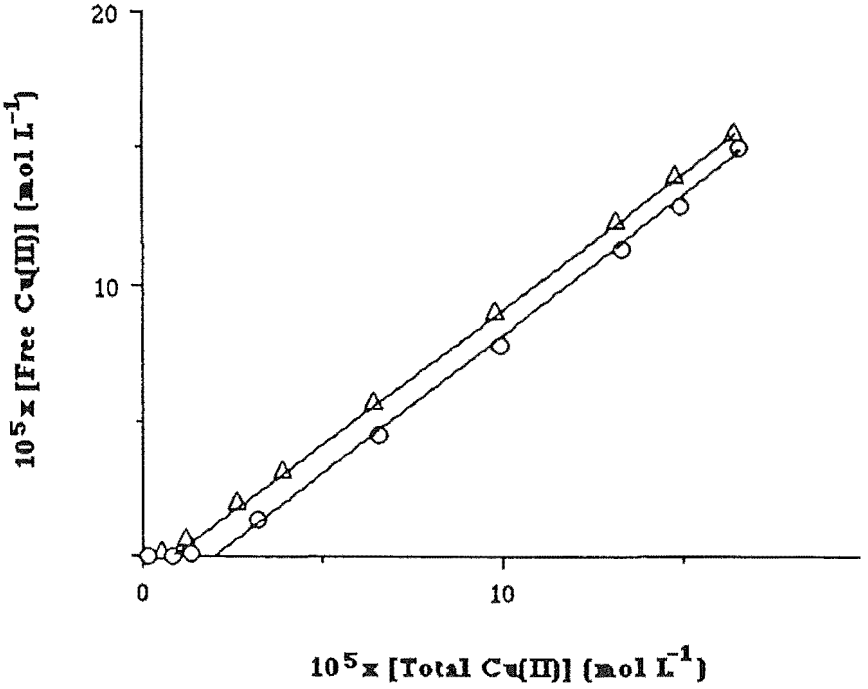


Figure 6.1a: Cu(II) Complexation Capacity Curves for Humic Acid

Δ pH 5.0; O pH 7.0.

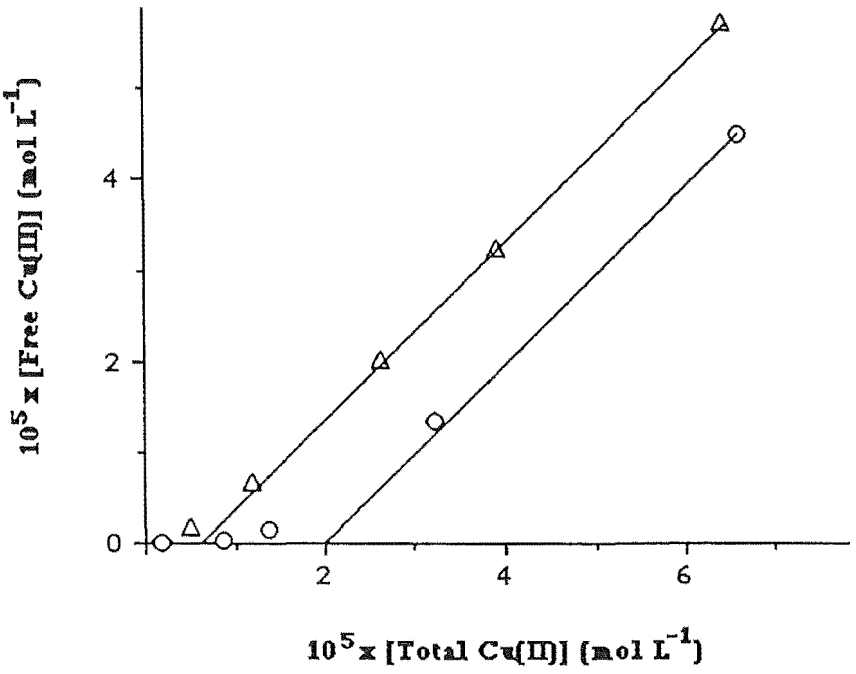


Figure 6.1b: Cu(II) Complexation Capacity Curves for Humic Acid

Δ pH 5.0, O pH 7.0

Assuming bidentate coordination (i.e. two carboxyl groups in each polydentate coordination site as in citrate and malonate) then, for fulvic acid at pH 5.0, 6.3, and 7.0 respectively, approximately 82 - 85%, 67 - 72%, and 50 - 60% of carboxyl groups were not involved in strong bonding under the experimental conditions. For humic acid (either unfiltered or filtered in alkaline solution) the proportions were 73 - 79%, 33 - 43%, and 5 - 25% respectively. The correction for Cu(II) hydrolysis becomes very large at pH 7.0, hence these data are subject to more error. The "free Cu(II) concentration" measured at this pH was greater than that allowable on the basis of the K_{sp} for $Cu(OH)_2$ (for which the concentration quotient is $10^{-19.36}$; Ganelina, 1964). However, stable EMF readings were obtained at pH 7, and despite the large correction for hydroxy species, the data exhibited remarkably good linearity. It is probable that a metastable system existed.

For humic acid which had been suspended in solution at pH 5.0 (i.e. not exposed to alkaline conditions) the complexation capacity for the unfiltered solution at pH 5.0 was the same as that for humic acid pre-dissolved in KOH. In contrast, that for a humic acid solution prepared then filtered at pH 5.0, but with the same final carboxyl group concentration, was only half that of the whole sample (91% of carboxyl groups not involved in complexing at pH 5.0), and 2.5 times less on the basis of weight of material present.

6.3.2 Copper(II) Binding Curves

Plots of percentage free Cu(II) *versus* pH at fixed Cu(II):ligand (COOH) ratios ([Cu(II)] = 4.0×10^{-5} and 9.0×10^{-6} mol L^{-1}) were constructed for fulvic and humic acid; Figures 6.2 and 6.3. These figures include the binding curves for humic acid measured at the same concentration by weight (mg mL^{-1}) as for the fulvic acid curves. Also given is the boundary calculated for precipitation of $Cu(OH)_2$ from a solution containing Cu(II) and its hydrolysis products.

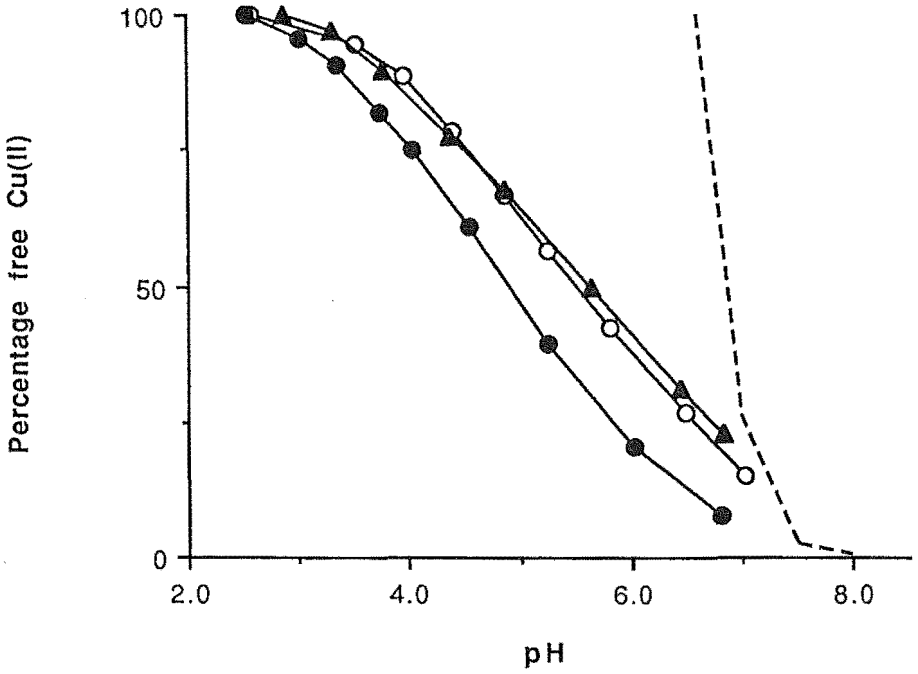


Figure 6.2: Cu(II) Binding Curves for Humic Substances

[Cu(II)] = 4.0 x 10^{-5} mol L^{-1}

● SHHA, [COOH] = 1.8 x 10^{-4} mol L^{-1}; O FA4, [COOH] = 1.8 x 10^{-4} mol L^{-1};

▲ SHHA at same concentration by weight as FA4; - - - - $Cu(OH)_2$ precipitation boundary.

Binding of Cu(II) by unfiltered and filtered (0.025 μm, at pH 12 and 5.0) humic acid was measured. The binding curves for the whole sample and that filtered at alkaline pH were the same, whereas that for humic acid filtered at pH 5.0 was displaced to higher pH (weaker binding); Figure 6.3.

Comparison of the binding curves for humic acid with those for fulvic acid indicated that the binding strength of these substances is similar on a weight basis. However, at the same carboxyl group concentration the humic acid curves were displaced markedly to lower pH, indicating stronger binding. For a 1:4.5 Cu(II):COOH ratio ([Cu(II)] = 4.0 x 10^{-5} mol L^{-1}) the pH displacement between the humic and fulvic acid curves was 0.53 at pH 3.5 and 0.63 at pH 5.0; for a 1:20 ratio ([Cu(II)] = 9.0 x 10^{-6} mol L^{-1}) the displacement was 0.65 and 1.0 respectively.

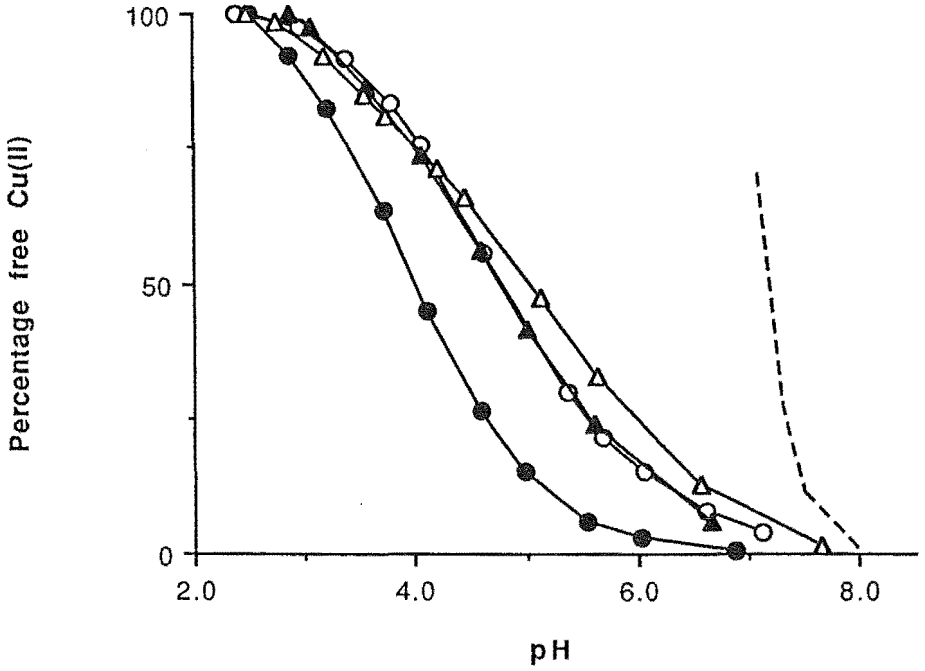


Figure 6.3: Cu(II) Binding curves for Humic Substances

[Cu(II)] = 9.0 x 10^{-6} mol L^{-1}

● SHHA, [COOH] = 1.8 x 10^{-4} mol L^{-1}; O FA4 [COOH] = 1.8 x 10^{-4} mol L^{-1};

▲ SHHA at same concentration by weight as FA4;

Δ SHHA 0.025 μm filtered at pH 5.0 at same concentration by weight as FA4;

- - - $Cu(OH)_2$ precipitation boundary.

Effect of Competing Ions on Cu(II) Binding Curves

Potassium

Results are given in Figure 6.4. In 0.60 mol L^{-1} KNO_3, the Cu(II) binding curve for FA4 (1:20 ratio) was displaced to higher pH (weaker binding). The displacement was 0.25 at pH 4.0 and 0.50 at pH 5.0 - 6.0. For a humic acid binding curve (at the same concentration by weight as FA4) the pH displacement was less, being 0.0 at pH 4.0, 0.19 at pH 5.0 and 0.38 at pH 6.0.

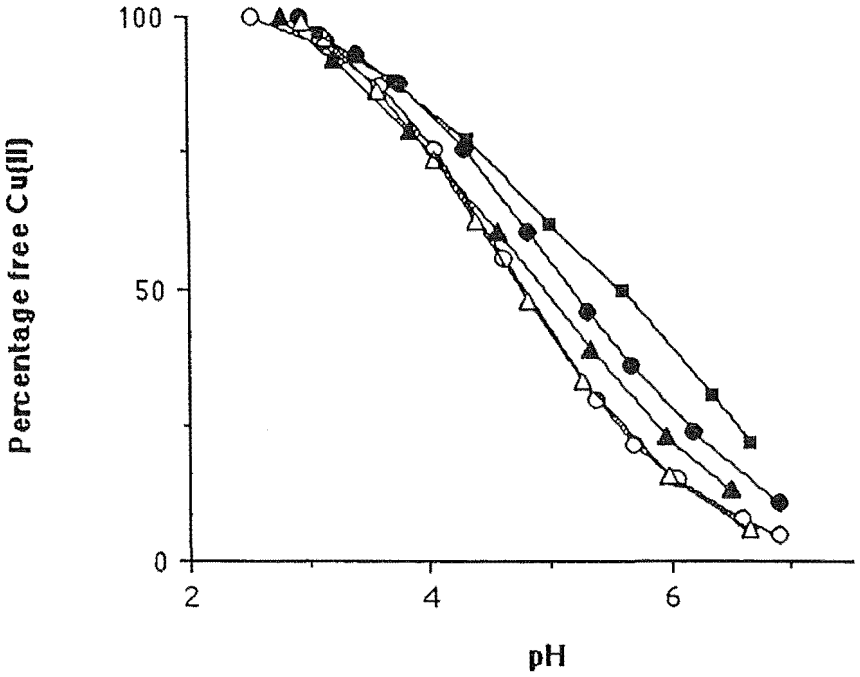


Figure 6.4: Cu(II) Binding Curves for Humic and Fulvic Acids: Effect of Potassium and Magnesium ([Cu(II)] = 9.0 x 10^{-6} mol L^{-1})

O FA4, [COOH] = 1.8 x 10^{-4} mol L^{-1} in 0.10 mol L^{-1} KNO_3; ● FA4 in 0.60 mol L^{-1} KNO_3;

■ FA4 in 0.06 mol L^{-1} Mg(II).

Δ SHHA at same concentration by weight as FA4, [COOH] = 8.7 x 10^{-5} M in 0.10 M KNO_3;

▲ SHHA in 0.60 mol L^{-1} KNO_3.

Mg(II)

The binding curve for FA4 at a 1:20 ratio was measured in the presence of 0.06 mol L^{-1} Mg(II) (a concentration equivalent to the concentration of divalent ions in seawater). The curve was displaced significantly to higher pH, by 0.38 at pH 4.0 and 1.0 at pH 5.0; Figure 6.4.

For comparison, the effect of Mg(II) and Ca(II) on Cu(II) complexation by citric acid was calculated from published stability constants. The species considered were: CuL (log β = 6.03), CuL_2 (10.43) (Ramamoorthy et al., 1972) MgL (3.40), MgHL (7.52), MgH_2L (5.19), CaL (3.55), CaHL (7.78), and CaH_2L (5.40) (Perrin, 1979). In a solution 1.8 x 10^{-4} mol L^{-1} in citrate and 9.0 x 10^{-6} mol L^{-1} in Cu(II), the percentage of Cu(II) bound to citrate was 54%, 98%, and 100% at pH 4.0, 5.0, and 6.0 respectively. In the presence of 0.053 mol L^{-1} Mg(II) and 0.01 mol L^{-1} Ca(II) (seawater composition) this

was reduced to 26%, 50%, and 53% respectively. This indicates that Ca(II) and Mg(II), when present in relatively high concentration, can compete effectively for Cu(II) complexation sites.

Al(III)

Binding curves for FA4 at a 1:4.5 ratio ([COOH] = 1.8×10^{-4} mol L^{-1}, [Cu(II)] = 4.0×10^{-5} mol L^{-1}) were measured in the presence of Al(III) (1.0×10^{-5} and 4.0×10^{-5} mol L^{-1}). The curves were displaced to higher pH in the region pH 3.5 - 6.5; Figure 6.5. In the presence of 1.0×10^{-5} mol L^{-1} Al(III) the pH displacement was 0.25 at pH 4.0 and 0.38 at pH 5.0; for 4.0×10^{-5} mol L^{-1} Al(III) the displacement was greater, being 0.38 and 0.51 respectively.

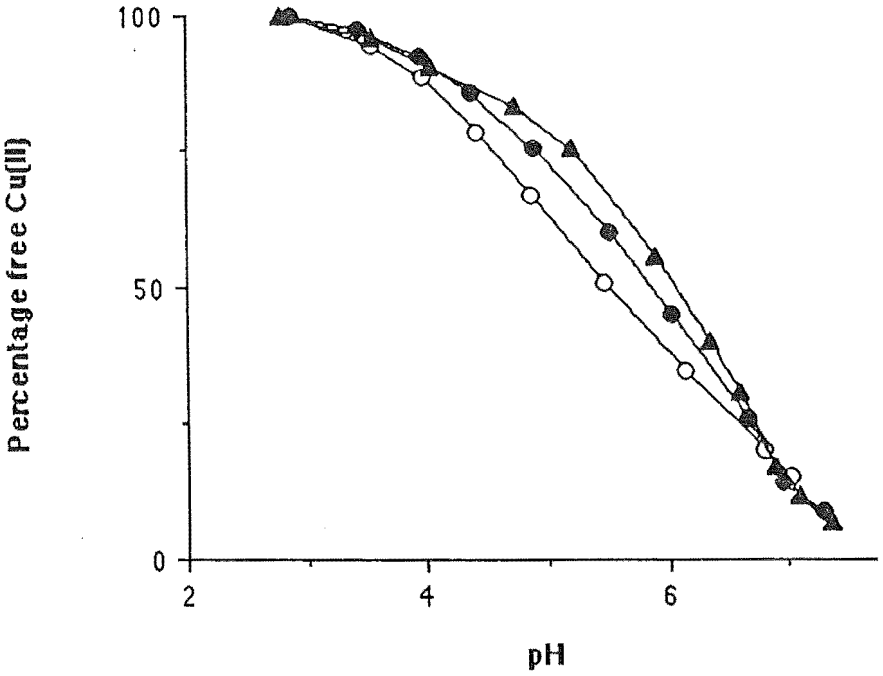


Figure 6.5: Cu(II) Binding Curve for Fulvic Acid: Effect of Al(III)

Cu(II): COOH = 1:4.5; [Al(III)] = 0.0 (O), 1.0×10^{-5} (●), and 4.0×10^{-5} mol L^{-1} (▲).

6.4 DISCUSSION

6.4.1 Calibration of the Copper(II) Ion Selective Electrode

Initial calibration of the ISE was against standard $Cu(NO_3)_2$ solutions in 0.10 mol L^{-1} KNO_3 ($p[H^+]$ *ca.* 4). The electrode response was linear in the range pCu 2.0 to 6.0 with Nernstian slope of 27.5 mV. For copper(II) concentrations less than 1.0×10^{-5} mol L^{-1}, *ca.* 30 min was required to obtain a stable reading in the absence or presence of humic substances at pH 5.0, 6.3, or 7.0; for Cu(II) concentrations greater than 3.5×10^{-5} mol L^{-1} the electrode potential was stable within 10 min.

Gregor (1987) established linearity in the range pCu 2.0 to 7.5 by calibration against citrate buffers. In the presence of citrate the Nernstian slope was reported to be the same as that for $Cu(NO_3)_2$ standards, but the intercept was shifted by +0.4 to +0.7 mV. Heijne and van der Linden (1978) reported calibration of a Cu(II) ISE to pCu 20 in Tetren and Trien buffers, while Avdeef et al. (1983) achieved calibration to pCu 19 in EDTA. In the presence of some complexing agents (e.g. EDTA and NTA) anomalous behaviour of a Cu(II) ISE has been reported (Heijne & van der Linden, 1978; Nakagawa et al., 1980).

In the present work, the Nernstian slope of the ISE was changed significantly in the presence of humic and fulvic acids (30.2 mV). Although this slope remained constant throughout these experiments, a continual drift in the intercept of the plot of EMF *versus* pCu (to more positive mV values) was observed over time. Therefore, for every ISE titration the electrode calibration was performed in the presence of ligand at pCu *ca.* 3.5, 4.0, and 5.0 at pH 2.5 - 2.8 (noncomplexing).

The Nernstian slope in the presence of humic substances (30.2 mV) was closer to the theoretical value of 29.6 mV than that obtained for the $Cu(NO_3)_2$ standards (27.5 mV). In the absence of ligand the Cu(II) solutions are poorly buffered especially at pCu 5 and 6. Although there was no evidence for curvature in the plots of EMF *versus* pCu for the $Cu(NO_3)_2$ standards it is likely that this data is less reliable. Fitch et al. (1986) have also reported Nernstian behaviour for a Cu(II) ISE in the presence of humic acid. In contrast, Sekerka and Lechner (1978) observed non-Nernstian behaviour and nonreproducibility in the presence of humic and fulvic acids. Using microprobe analysis,

Buffle et al. (1977) found no evidence for adsorption of humic or fulvic acids on the surface of Cu(II), Pb(II), and Cd(II) ISEs.

Anomalous behaviour was observed if the ISE was calibrated at pH < 2.5 (the apparent concentration of free Cu(II) was enhanced). In this pH region the electrode also responds to hydrogen ions (Buffle et al., 1980). Barica (1978) reported enhanced response of a Cu(II) ISE in alkaline (pH 7.8 - 8.6), moderately saline waters. Wagemann (1980) reported that in addition to free Cu(II) ions, an ISE responds to $Cu(OH)^{+}$, $Cu_2(OH)_2^{2+}$, and $CuHCO_3^{+}$ above pH 7. In the present work, the slope of the linear portion of the complexation capacity curves was considerably less than 1.0 if it was assumed that the electrode responds to both free Cu(II) and the hydroxy species. Including a correction for hydroxy species in calculation of the "free Cu(II) concentration" did give a slope ≈ 1, suggesting that the ISE was not responding to these species.

Two types of experiments were performed, *viz:* metal binding curves (plots of percentage free Cu(II) *versus* pH) and complexation capacity curves (plots of free Cu(II) *versus* total added Cu(II)).

The curves for fulvic acid were compared with those for humic acid (both unfiltered and 0.025 μm membrane filtered) on the basis of both the carboxyl group content and the weight of material. The equivalent weight of SHHA was 287, that of FA4 was 139 (Chapter 3). Hence for the same weight concentration of humic substance, the carboxyl group content of a SHHA solution is *ca.* half that of FA4.

In the titrations involving humic acid a solid phase was present. Hence, the modes of binding available may well be different from those in solution of the more soluble fulvic acid. No attempt was made to take this into account. It is noted that precipitation could also occur on complexation of Cu(II) by fulvic acid (Schnitzer & Kerndorff, 1981; Piccolo & Stevenson, 1982; Gamble et al., 1985).

6.4.2 Copper(II) Complexation Capacity Curves

Extrapolation of the linear portion (slope ≈ 1) of plots of free Cu(II) *versus* total added Cu(II) was taken as a measure of the complexation capacity (Figure 6.6). This technique has been used by other workers (e.g. Truitt & Weber, 1981; Rainville & Weber, 1982; van Leeuwen et al., 1989b). However, some authors have used the intersection of the tangents of the "two linear sections of the graph" to determine the complexation capacity of humic substances (e.g. Hart, 1981; Varney et al., 1984; Florence, 1986). In the present work, titration of discrete ligands, such as citrate, established that extrapolation of the linear portion of the curve, with slope ≈ 1, gives the correct complexation capacity.

The inherent metal content of humic substances must be considered when comparing complexation capacities for different samples (Cressey et al., 1983; Midorikawa, 1990). Low-ash humic samples were used in the present work (Chapter 3).

The lower proportion of fulvic acid carboxyl groups involved in strong bonding with Cu(II) (less than half of the total at pH 7.0 assuming bidentate carboxyl coordination) is consistent with a high proportion of structurally isolated carboxyl groups (nonchelating moieties), stereochemically inaccessible carboxyl groups, or groups which are weak complexors below pH 7.0 (e.g. salicylate).

At pH 5.0, citric acid was considered a good model for fulvic acid complexation capacity curves (Gregor, Powell & Town, 1989a); this was also observed for humic acid in the present work. The capacity curves for fulvic and humic acids at pH 6.3 are compared with those calculated for model ligands in Figures 6.6 and 6.7. At this pH the more weakly binding malonic acid is an appropriate model for both the fulvic and humic acid curves. The concentration of model ligands used for these simulations was chosen to be equivalent to the measured complexation capacity for the humic substances.

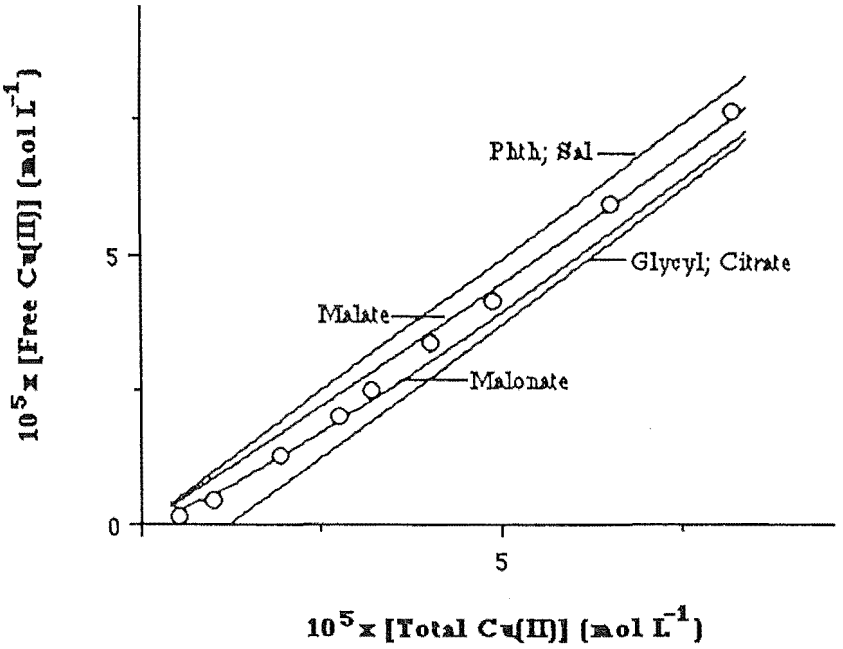


Figure 6.6: Complexation Capacity Curves for Fulvic Acid and Model Ligands, pH 6.3.

O FA4, [COOH] = 5.95 x 10^{-5} mol L^{-1}; [model ligands] = 1.30 x 10^{-5} mol L^{-1}.

Abbreviations: Phth = phthalate; Sal = salicylate; Glycyl = glycylaspartate.

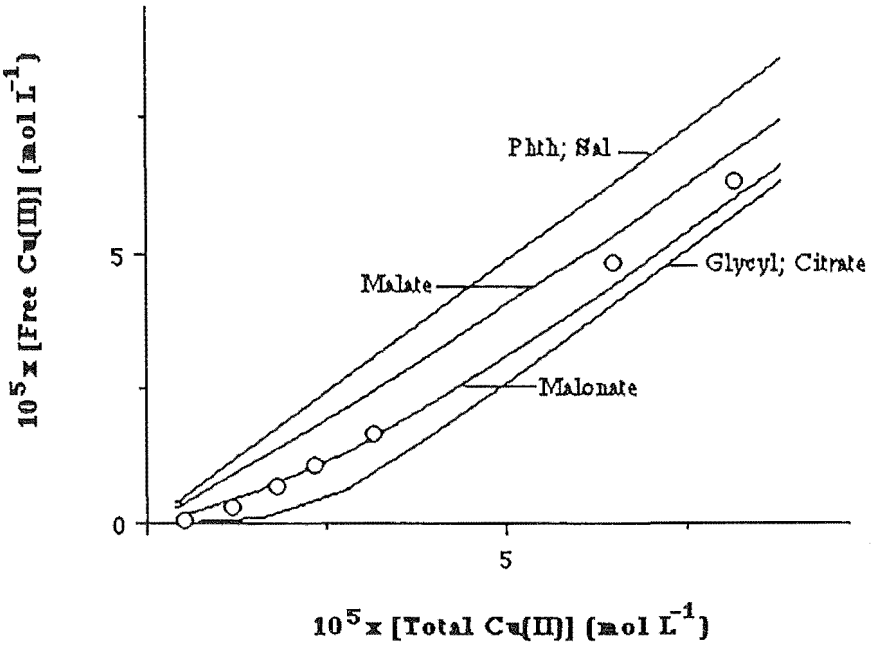


Figure 6.7: Complexation Capacity Curves for Humic Acid and Model Ligands, pH 6.3

O SHHA, [COOH] = 5.58 x 10^{-5} mol L^{-1}; [model ligands] = 2.47 x 10^{-5} mol L^{-1}.

Abbrevations as per Figure 6.6.

The heterogeneous nature of humic substances means that the coordination mode is likely to change with pH and with metal:ligand ratio. The observation that different discrete ligands more closely model sections of the humic substance curves at different pH values supports this hypothesis.

For humic acid the proportion of carboxyl groups involved in strong Cu(II) binding was *ca.* 1.5 times greater than that for fulvic acid at pH 5.0 and *ca.* 2.0 times greater at pH 6.3 and 7.0. This observation is consistent with humic acid carboxyl groups having more favourable configurations for Cu(II) complexation than those of fulvic acid.

On a weight basis, the complexation capacity for humic acid (unfiltered or filtered in alkaline solution) was similar to that for fulvic acid, *viz: ca.* 1.0×10^{-3} and 1.2×10^{-3} moles Cu(II)/g humic substance at pH 6.3 and 7.0 respectively. Most methods reported for the determination of Cu(II) complexation capacities give values between 0.3×10^{-3} to 3.0×10^{-3} mol Cu/g humic substance (Buffle et al., 1984).

The complexation capacity for a humic acid sample which was filtered in alkaline solution was the same as that for an unfiltered solution. This implies that the moieties which are insoluble at pH 12 do not contribute significantly to strong Cu(II) binding (Sojo et al., 1989).

The effect of dissolution of humic acid under milder conditions was also investigated. The complexation capacity at pH 5.0 for unfiltered humic acid which had been equilibrated at pH 5.0 was the same as that for a solution which had been predissolved in 0.8 mol L^{-1} KOH. This result indicates that exposing the humic acid to strongly alkaline conditions did not significantly alter the functional groups involved in strong Cu(II) binding. However, the complexation capacity for a humic acid sample which had been equilibrated at pH 5.0 then 0.025 μm membrane filtered at this pH was less than half that for the unfiltered sample on the basis of both the carboxyl group content and the weight of material present. This implies that a considerable amount of the complexation capacity of soils (and possibly natural waters) may be associated with the colloidal/particulate phase. Further, it indicates that the solid humic acid phase does strongly complex Cu(II). Studies on the solubility and molecular size fractionation of humic acid as a function of pH (Chapter 5) established that predominantly smaller molecules would be soluble at pH 5.0.

In making comparisons between filtered and unfiltered humic acid samples it was assumed that these fractions had the same equivalent weight. This may not be a valid assumption. For example, Collins et al. (1986) stated that the carboxyl content of humic substances was inversely proportional to their molecular weight. Similarly, Kim et al. (1990) and Dell'Agnola and Ferrari (1971) have reported a lower carboxylate content for larger humic molecules (>70 000 Dalton).

The increase in complexation capacity on raising the pH from 5.0 to 6.3 was greater for humic acid than for fulvic acid. Hence, humic acid may contain a greater proportion of moieties which deprotonate in the higher pH range (e.g. salicylate) and/or the humic acid moieties may undergo some conformational change over this pH range which generates more effective coordination sites (Bresnahan et al.,1978; Bonnett & Cousins, 1987).

It has been reported that complexation capacities for humic substances vary with pH, ionic strength, concentration of humic substance, and the nature of the metal ion (Sanders & Bloomfield, 1980; Truitt & Weber, 1981; Fitch et al., 1986). However, according to Perdue (1989) these observations are an artifact of the experimental procedure. Perdue (1989) proposed that the complexation capacity of humic substances is approximately equal to their total exchangeable acidity and that "the extent to which this complexation capacity can be realized in experimental measurements is strongly a function of pH, ionic strength, nature of the metal, and the concentration of humic substances used in the measurement". In the present work, the Cu(II) complexation capacity of fulvic acid was not altered by a three-fold change in the concentration of humic substance, nor by an increase in ionic strength from 0.10 to 0.60 mol L^{-1} KNO_3. Hering and Morel (1988a) reported no change in the Cu(II) complexation capacity of humic acid in the presence of 0.01 mol L^{-1} Ca(II).

6.4.3 Copper(II) Binding Curves

The Cu(II) binding curves for humic and fulvic acids were compared on the basis of carboxyl group content and the weight of material present. Both unfiltered and 0.025 μm membrane filtered humic acid samples were studied; the effect of competing metal ions was also investigated. Attempts were made to model these curves using combinations of discrete ligands.

Comparison of Cu(II) Binding by Humic and Fulvic Acids

The curves for all humic substances were displaced to higher pH (weaker complexing) at the higher Cu(II) concentration (4.0×10^{-5} mol L^{-1}) indicating the heterogeneity of available chelation sites. A much smaller displacement was observed for the model ligands. The most strongly binding sites will dominate complexing at low metal-to-ligand ratios, followed by progressively weaker binding sites at higher concentrations of Cu(II) (Sanders & Bloomfield, 1980; Perdue, 1989)

On the basis of carboxyl group content the binding curves for humic acid (1:4.5 and 1:20 ratio) were displaced significantly to lower pH compared to those for fulvic acid, indicating stronger binding; Figures 6.2 and 6.3. This indicates that the carboxyl groups in humic acid have more favourable configurations for strong Cu(II) complexation and/or that humic acid contains functional groups, other than carboxyl, which strongly complex Cu(II).

The binding curves for unfiltered humic acid and that filtered in alkaline solution were identical, indicating that the molecules which are insoluble at pH >12 do not contribute significantly to Cu(II) binding over the pH range 2.5 - 7.0.

On the basis of weight of material in solution (mg mL^{-1}) the binding curves for fulvic and humic acids were very similar (FA, [COOH] = 1.8×10^{-4} mol L^{-1}; SHHA, [COOH] = 8.5×10^{-5} mol L^{-1}).

For unfiltered humic acid which had been equilibrated at pH 5.0, the Cu(II) binding curve was similar to that for a sample predissolved in KOH. This indicates that exposure to a strongly alkaline solution did not further alter the Cu(II) complexation sites (its complexation capacity was also not affected; Section 6.4.2). It is noted that the humic acid was originally extracted with 0.1 mol L^{-1} NaOH. However, for a humic acid sample

equilibrated at pH 5.0 and 0.025 μm membrane filtered at this pH, the binding curve was displaced to higher pH, indicating weaker binding (Figure 6.3). The pH displacement was 0.19 at pH 4.5, and 0.33 at pH 6.0. At pH 5.0 predominantly smaller humic acid molecules will be in solution (Chapter 5) and these moieties may have a higher carboxyl group content (Dell'Agnola & Ferrari, 1971; Collins et al., 1986). Therefore, this result indicates that the larger humic acid molecules are stronger complexors for Cu(II) than are the smaller components, or fulvic acid. This has important implications in terms of speciation. The particulate/colloidal organic phase may be the predominant sink for metal ions in the environment. It may also be the fraction most involved in moderating toxicity to biota and buffering influxes of contaminants into soils and natural waters.

Although it is often claimed that humic acids complex metal ions more strongly than do fulvic acids (e.g. Shanmukhappa et al., 1986), the author could not find much evidence reported to substantiate this statement. A study by Young et al. (1982) found stronger binding of Cu(II) by higher molecular weight humic substances. These authors reported that organic matter with a greater density of carboxyl groups complexes Cu(II) less strongly than did other fractions. It was argued that a high carboxyl group density was accompanied by an increase in other types of functional groups which exert a chemical dipole influence over neighbouring carboxyl groups producing lower protonation constants and discouraging cation binding (Young et al., 1981). As noted above, this result could also indicate carboxyl groups which are structurally isolated or stereochemically unfavourable for coordination. Alternatively, there may be other moieties in humic acid which facilitate Cu(II) coordination. It has been proposed that the carboxyl and phenolic groups in humic acids may be distributed in different molecular weight or structural fractions than in fulvic acids (Sojo et al., 1989).

Erroneously, it has been claimed that fulvic acids would have the higher affinity for metal ions because they contain a higher proportion of carboxylic and phenolic hydroxyl groups than do humic acids (Hansen et al., 1990).

Reversibility of Binding Curves

The reversibility of the binding curves was measured for humic and fulvic acid at a 1:20 Cu(II):COOH ratio.

Addition of HNO_3 to a Cu(II)-fulvic acid solution which had stood at pH 7 overnight generated the same binding curve as was obtained for addition of KOH to an acidic solution of Cu(II) and fulvic acid. In contrast, for a Cu(II)-humic acid solution which had stood at pH 7 overnight the EMF obtained for the reverse titration was 2.6 mV more positive at pH 3 than that for the original acidic solution; for a solution immediately acidified, the EMF was 1.2 mV more positive.

A greater EMF value indicates a lower free Cu(II) concentration. Therefore, the above result for humic acid may represent the formation of nonlabile Cu(II)-humic acid species which do not dissociate on acidification.

Nonlabile Humic Substance Complexes

Buffle et al. (1977) reported an increase in the degree of complexation in a Cu(II)-fulvic acid solution which had stood at pH 8 overnight; no effect was observed if the forward and back titrations were performed over 2 - 3 h. Further, no hysteresis was observed if the titrations were carried out at pH <7. Buffle et al. (1977) reasoned that the nonlabile Cu(II)-fulvic acid fraction resulted from adsorption of hydrolyzed metal on colloidal organic matter or from formation of mixed OH-Cu-FA complexes. Slowly reacting sites could also explain this hysteresis. For example, Lavigne et al. (1987) studied the kinetics of Ni(II) complexation by a soil fulvic acid. Following equilibration at pH 4 or 5, complete recovery of Ni(II) could be attained. However, at pH 6.4 40% of the Ni(II) was bound in a nonlabile form which required 10 days for complete recovery. Interestingly, an equivalent effect was not observed for aquatic humic substances (Cabaniss, 1990). Cu(II) complexation by cyclic porphyrin structures could result in slow reactions (Cabbiness & Margerum, 1970). Indeed, it has been proposed that humic acid behaves as a macrocylic ligand towards metal ions, thus forming very stable complexes (Nissenbaum & Swaine, 1976). Evidence has been reported for copper(II) porphyrin-type complexes persisting in humic (but not fulvic) acids treated with strong acid (Goodman & Cheshire, 1973, 1976; Cheshire et al., 1977). Recently, Caceci and Billon

(1990) have identified large associations of molecules in humic acid samples (0.05 - 0.2 μm in diameter). These authors suggested that metals complexed by such structures would be difficult to remove.

Fischer (1986) reported partially irreversible complexation of Cu(II), Pb(II), and Cd(II) by aqueous humic substances and proposed that very stable metal species were associated with solid humic phases. Studies on the equilibration of metals with humic acids have indicated that a significant proportion of the metal is not recoverable by acidification, or by ligand exchange (Sequi et al., 1975; Slavek et al., 1982; Mak & Langford, 1982).

The formation of such nonlabile species could serve as a sink for metal ions in soils and natural waters.

6.4.4 Simulation of Binding Curves

Erroneously, it has been assumed that the functional groups in humic substances which are numerically dominant (salicylate and phthalate) will control metal complexing (Murray & Linder, 1983; Goncalves & Mota, 1987). However, it has been demonstrated that humic substances complex Cu(II) much more strongly than do these moieties (Cheam, 1973; Cheam & Gamble, 1974; Buffle et al., 1977; Midorikawa et al, 1990). The binding curves for FA4 and SHHA are compared with those for discrete model ligands in Figures 6.6 to 6.9. The concentration of model ligands used for these simulations was chosen such that their complexation capacity was equivalent to that for a humic substance solution at pH 6.3. The much weaker complexation by salicylate is clearly demonstrated. Malonate is the best model for fulvic acid binding at both metal-to-ligand ratios (Figures 6.6 and 6.7); however, it is a poor fit to the data above pH *ca.* 5 (moieties such as glycylaspartate and MHBS may then contribute to complexing). For humic acid, complexation at 9.0×10^{-6} mol L^{-1} Cu(II) (strongest binding sites utilized) is stronger than for any of the model ligands considered (Figure 6.9). At the higher Cu(II) concentration the binding curve is reasonably well modelled by malonate up to pH *ca.* 6 (Figure 6.8).

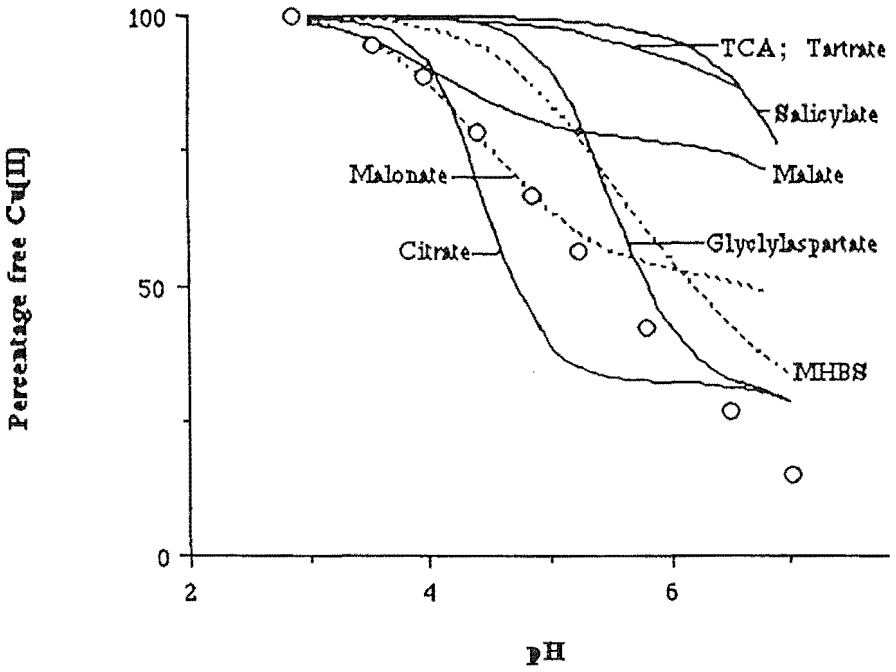


Figure 6.6: Cu(II) Binding Curves for Fulvic Acid and Model Ligands

[Cu(II)] = 4.0 x 10^{-5} M

O FA4, [COOH] = 1.8 x 10^{-4} mol L^{-1}; [Model ligands] = 2.7 x 10^{-5} mol L^{-1}.

Abbreviations: TCA = tricarballylic acid; MHBS = 5-methoxy-N-(2-hydroxybenzyl)sarcosine.

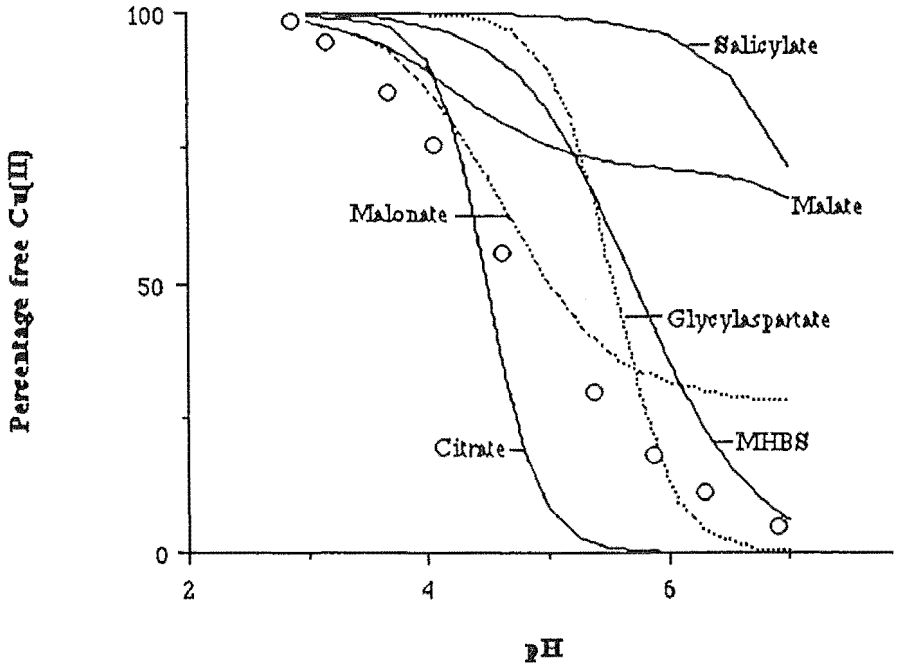


Figure 6.7: Cu(II) Binding Curves for Fulvic Acid and Model Ligands

[Cu(II)] = 9.0 x 10^{-6} M

O FA4, [COOH] = 1.8 x 10^{-4} mol L^{-1}; [Model ligands] = 2.7 x 10^{-5} mol L^{-1}.

Abbreviations as per Figure 6.6.

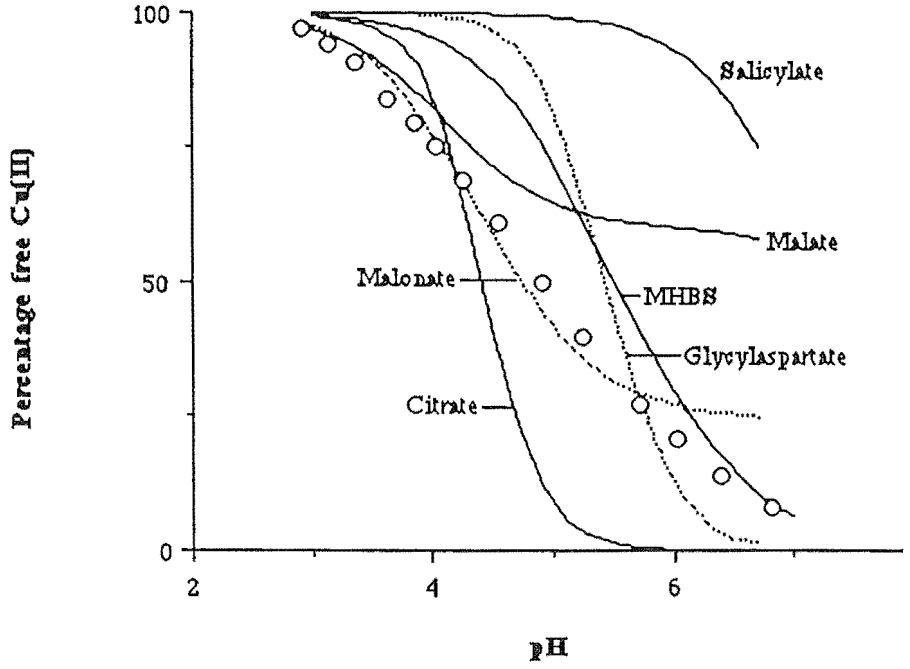


Figure 6.8: Cu(II) Binding Curves for Humic Acid and Model Ligands

[Cu(II)] = 4.0 x 10^{-5} M

O SHHA, [COOH] = 1.8 x 10^{-4} mol L^{-1}; [Model ligands] = 5.58 x 10^{-5} mol L^{-1}.

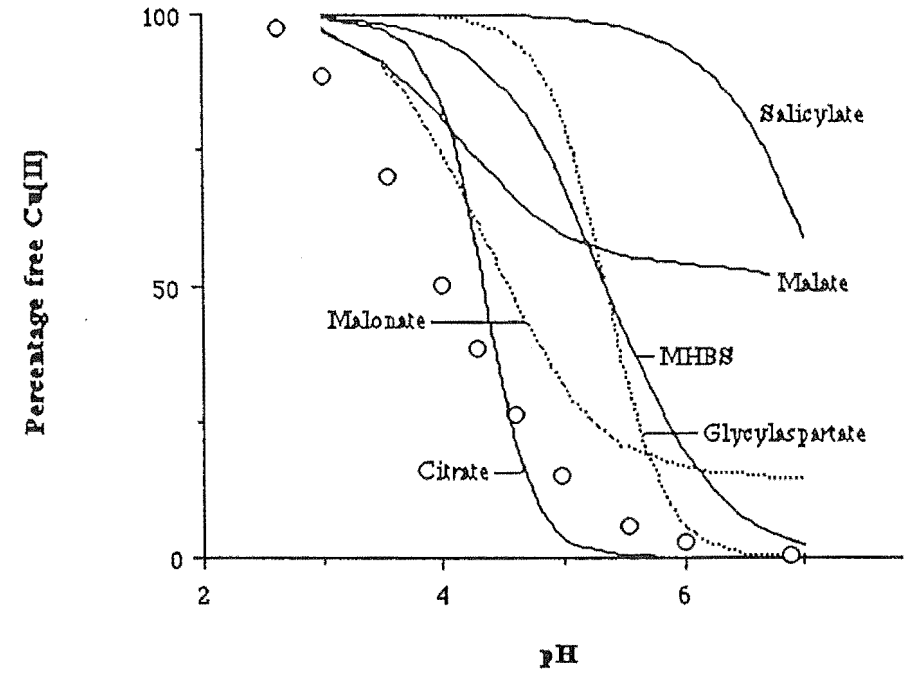


Figure 6.9: Cu(II) Binding Curves for SHHA and Model Ligands

[Cu(II)] = 9.0 x 10^{-6} M

O SHHA, [COOH] = 1.8 x 10^{-4} mol L^{-1}; [Model ligands] = 5.58 x 10^{-5} mol L^{-1}.

No single discrete ligand moiety could adequately describe the humic substance binding curves over the entire pH range. A more realistic model would involve several ligand moieties competing for available metal ions.

Initial attempts to model fulvic acid binding curves (Gregor, Powell & Town, 1989a) established that a combination of citric, tricarballylic, aspartic, malonic, and salicylic acids with respective concentrations: (i) 0.36 x 10^{-6}, 1.62 x 10^{-5}, 2.05 x 10^{-5}, 2.1 x 10^{-5} and 2.1 x 10^{-5} mol L^{-1}; or, (ii) 0.90 x 10^{-5}, 1.62 x 10^{-5}, 2.05 x 10^{-5}, 2.05 x 10^{-5} and 0.0 mol L^{-1} provided reasonable models for the fulvic acid data. Although these model ligand compositions were consistent with the amino acid nitrogen content of fulvic acids, they contained a higher proportion of mono plus diprotic moieties than was allowable by the pK_a spectrum for fulvic acid (Gregor & Powell, 1988b). A basic model consistent with the fulvic acid pKa spectrum may include: (i) a tetracarboxylic acid representing 8% of ligand moieties, pK_4 = 6.5 (modelled by tricarballylic acid as the stability constants for butane-1,2,3,4-tetracarboxylic acid could not be determined (Chapter 4)); (ii) a triprotic acid (e.g. citric, pK_3 = 5.7) or 1,1'-diprotic acid (e.g. malonic, pK_2 = 5.4) at 4%; (iii) a diprotic acid (e.g. malic, pK_2 = 4.46) at 8%; and (iv) a monoprotic acid (e.g. salicylic, pK_1 = 2.9) at 40%.

However, a model consistent with this criterion resulted in a poorer fit to the data (Gregor, Powell & Town, 1989b). On comparison with Cu(II) binding curves for fulvic acid, a solution composition of: citric acid, 0.72 x 10^{-5} mol L^{-1}; tricarballylic acid, 1.44 x 10^{-5} mol L^{-1}; peptide (glycylaspartic acid), 0.24 x 10^{-5} mol L^{-1}; malic acid, 1.44 x 10^{-5} mol L^{-1}; and salicylic acid, 7.2 x 10^{-5} mol L^{-1} incorporated insufficient strongly binding sites, and the sum of these was less than the measured complexation capacity for fulvic acid. Additional moieties were required which bind Cu(II) strongly and which deprotonate at pH >5.5.

A problem with these models is that moieties containing functional groups other than carboxyl may also contribute to Cu(II) complexing. For example, the hydroxyl content of fulvic acids is significant; thus, enolic moieties may contribute additional binding sites (Ephraim et al., 1989). Indeed, inclusion of acetylacetone

(1.6×10^{-5} mol L^{-1}) in the model significantly improved the fit to the fulvic acid data (Gregor, Powell & Town, 1989b).

For these earlier attempts to simulate fulvic acid binding curves the total carboxyl concentration for the mixture of model ligands was set at the total fulvic acid carboxyl concentration (1.8×10^{-4} mol L^{-1}). However, the present work has established that on the basis of complexation capacity measurements, comparison of Cu(II) binding curves for humic substances and model ligands should consider carboxyl group concentrations significantly below the total humic substance carboxyl content. Further, the Cu(II) complexation capacity of humic substances increased as the pH increased (from *ca.* 8% of the total carboxyl group concentration at pH 5.0 to 22% at pH 7.0 for fulvic acid; and from *ca.* 12% to 42% respectively for humic acid). These factors should be included in models used to simulate these curves *via* mixtures of discrete model ligands.

Table 6.1: Models for the Simulation of Fulvic Acid Binding Curves

Ligand	Model A[a]	Model B[a]	Model C[a]	Model D[a]
Citrate	0.72	0.72	0	0.72
Tricaballylate	1.44	1.44	1.44	1.44
Glycylaspartate	0.24	0.00	0.00	0.00
Malate	1.44	1.44	1.44	1.44
Salicylate	7.20	7.20	7.20	7.20
Acetylacetone	1.60	1.60	1.60	1.60
Malonate	0.00	0.00	1.08	0.00
MHBS[b]	0.00	0.24	0.24	0.80

[a] $10^5 \times$ mol L^{-1}.

[b] 5-methoxy-N-(2-hydroxybenzyl)sarcosine.

The previous model of 'best fit' to the fulvic acid data (Model A, Table 6.1) was altered slightly in the present work. Specifically, the effect of substituting MHBS for glycylaspartic acid, and malonic acid for citric acid was investigated.

The combinations of discrete ligands used and their resulting binding curves are given in Table 6.1 and Figure 6.10 respectively.

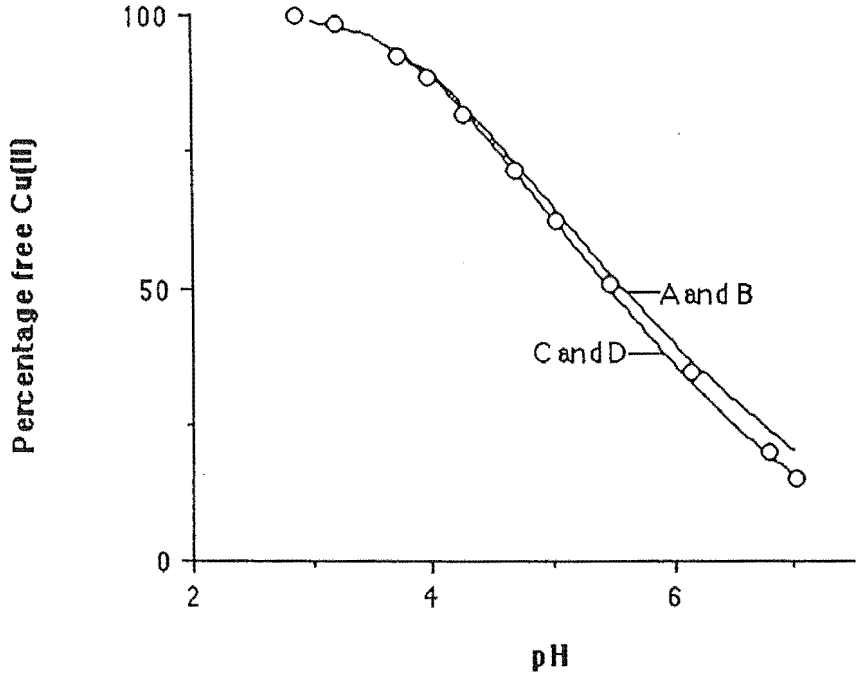


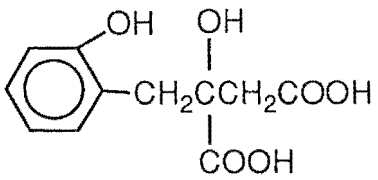
Figure 6.10: Cu(II) Binding Curves for Fulvic Acid and Mixtures of Model Ligands: [Cu(II)] = 4.0 x 10^{-5} M

O FA4, [COOH] = 1.8 x 10^{-4} mol L^{-1}; Curves A, B, C, D defined in Table 6.1.

The replacement of citrate with malonate (at an equivalent carboxyl concentration) had no effect on the calculated curve (Curves C and D, Figure 6.10). Models C and D provide an excellent fit to the fulvic acid data (at both Cu(II) concentrations). The primary modification of the previous model was an increase in the concentration of amino acid moieties to a value equivalent to 100% of the typical characterized amino acid content of fulvic acids; previously this was set at 30% (Gregor, Powell & Town, 1989b). This result indicates the critical role that nitrogen containing moieties may have in determining metal coordination by humic substances. Electron spin resonance studies have provided evidence for strong complexation of Cu(II) by humic substances involving 3 O, 1 N, or 2 O, 2 N atoms arranged in a square planar environment (Senesi et al., 1985, 1989). Hoffman et al. (1981) proposed that macrocyclic ligands such as porphyrins and phthalocyanines (which bind metal ions *via* pyrrole nitrogen atoms) may play an important role in the complexation of metals in natural waters. These authors noted that

the apparent stability of Cu(II) complexes in natural waters cannot be ascribed to carboxylate ligands alone.

Another factor may be 'cascade binding'. That is, isolated weakly complexing groups, e.g. phenolic or aliphatic hydroxyl, may contribute to binding *via* new 7 or 8 membered chelate rings. Fluorescence quenching measurements have been reported which support this concept (Gregor, Powell & Town, 1989a,b). (Substituted aromatic moieties are the most likely fluorescent centres in humic substances.) These fluorescence quenching data could be explained by either polydentate fluorescent moieties being involved in Cu(II) complexation, or by the fluorescent centres being within 1 or 2 carbon atoms of the primary binding sites. A structural unit consistent with the latter interpretation is:



where the primary binding site is a citrate like moiety. It is possible that cascade binding makes a significant contribution to strong Cu(II) complexation in the pH region above pH 5.5. Complexing by polycarboxylate ligands is essentially complete below this pH and additional binding must involve groups with low acidity, such as phenols or amino acid nitrogen.

To probe the possible contribution of cascade binding to Cu(II) complexation by humic substances a quantitative study of the Cu(II)-MHBS system was performed (Chapter 4). It was demonstrated that the proximity of a phenolic hydroxyl group (in itself weakly binding) to a strongly coordinating amino acid moiety promoted formation of a stable complex (*via* a 6 membered chelate ring).

Simulation of the Cu(II) binding curves for humic substances was attempted including MHBS as a model ligand. Substitution of MHBS for glycylaspartic acid had no effect on the calculated binding curve (compare curves A and B, Figure 6.10). However, because MHBS (and glycylaspartate) contain an amino acid moiety the concentration at which these could be included in the calculations was limited. Further, cascade binding in Cu(II)-MHBS is effective at relatively low pH. For a ligand which forms a 7 or 8

membered chelate ring on coordination with Cu(II) the stability constant will be lower, i.e. the reaction will occur at higher pH. The inclusion of such non-nitrogen moieties at significant concentrations could have a measurable impact on the Cu(II) binding curves. Unfortunately, to the author's knowledge such ligands or synthetic routes to such ligands were not available for use in these studies.

A factor not previously considered is the percentage of carboxyl groups which are involved in Cu(II) complexation in the mixed model ligand solutions at each pH. The percentage of each ligand complexed as a function of pH for Model D (Table 6.1) is given in Figures 6.11 and 6.12. At 9.0 x 10^{-6} mol L^{-1} Cu(II) the percentage of tricarballylate bound to Cu(II) reached a maximum of 0.43 at pH 5.8; the percentage of salicylate coordinated was 0.46 at pH 7.0.

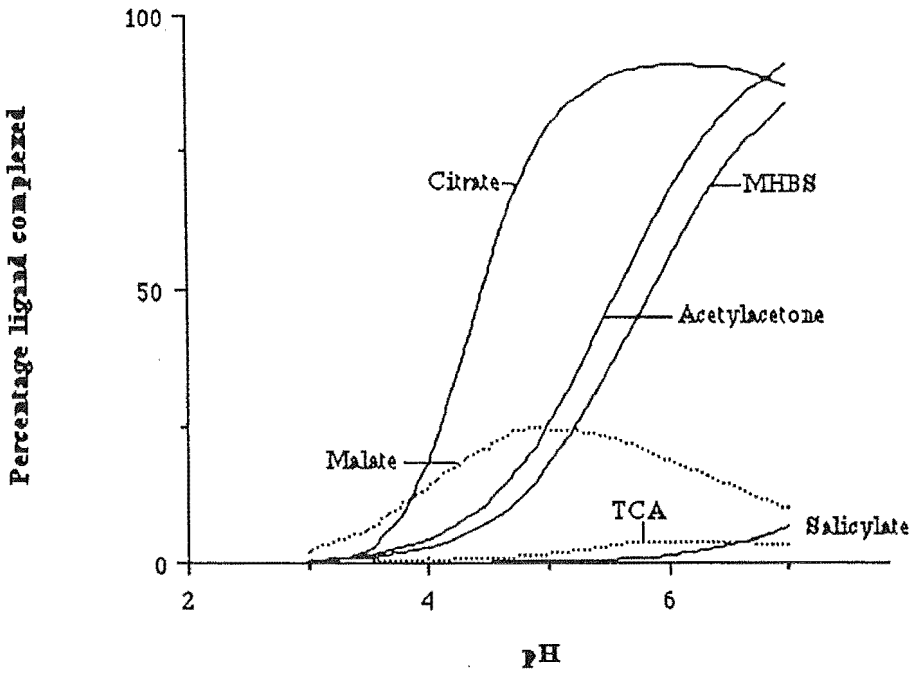


Figure 6.11: Distribution of Species for Model D: [Cu(II)] = 4.0 x 10^{-5} M

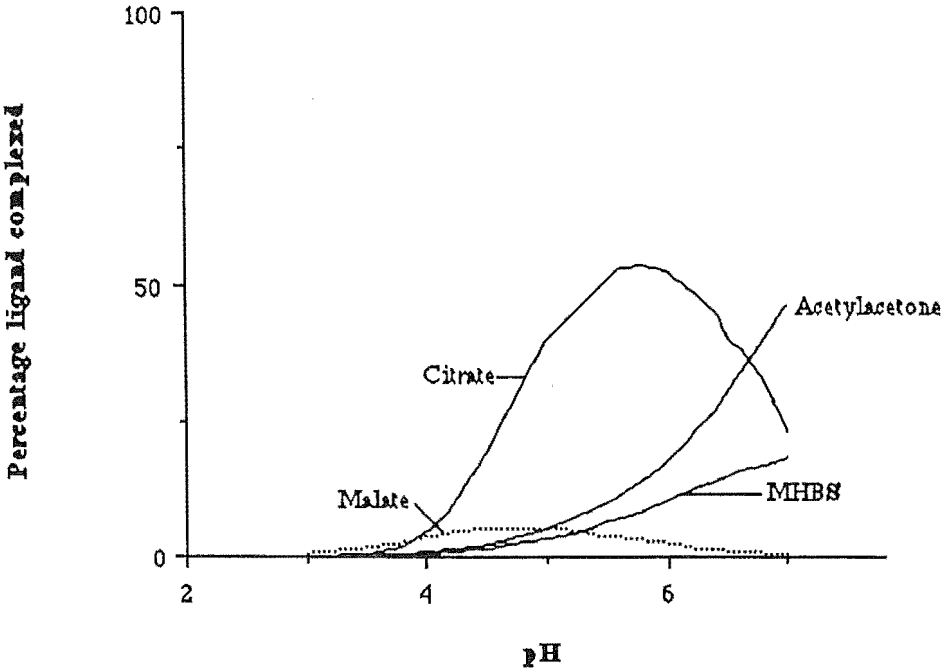


Figure 6.12: Distribution of Species for Model D: [Cu(II)] = 9.0 x 10^{-6} M

This illustrates that in a mixture of discrete ligands, having different stability constants, the proportion of each donor group involved in complexing will change with pH. For this model ligand composition at a 1:4.5 ratio, 15% of the total carboxyl groups were involved in complexing at pH 5.0 and 18% were utilized at pH 6.3. Hence, for this model ligand composition at a 1:4.5 ratio the percentage of carboxyl moieties involved in complexation at each pH is reasonably consistent the complexation capacities measured for fulvic acid. In contrast, at the lower Cu(II) concentration (9.0 x 10^{-6} mol L^{-1}) only 6% of carboxyl groups were utilized at pH 5.0 and 7.0. It is noted that the complexation capacity is measured in the presence of an excess of Cu(II).

Figures 6.11 and 6.12 illustrate an important concept; the proportion of carboxyl groups involved in complexing varies with metal-to-ligand ratio. The *maximum* proportion of carboxyl groups which may participate in Cu(II) complexation at a given pH is determined by the complexation capacity curves; a lower amount will be involved at lower metal-to-ligand ratios (i.e. for the binding curves). A 1:4.5 ratio in the complexation capacity curves occurs at the beginning of the linear portion of the graph (slope $\approx$ 1); hence, the proportion of carboxyl groups utilized at this metal-to-ligand ratio is reasonably consistent with the complexation capacity measurements.

For humic acid the complexation capacity was greater (by a factor of 1.7 at pH 6.3) than that calculated on the basis of the percentage of carboxyl groups involved in complexation in the mixture of discrete ligands (Figure 6.11). This is in contrast to fulvic acid. This indicates that there are some functional groups in humic acids which are not adequately modelled by the discrete ligands. Alternatively, 'enhanced coordination' of carboxyl groups may occur in humic acids and/or the humic molecules could undergo some conformational changes as the pH increases. Therefore, to simulate the humic acid curves, the concentration of ligand moieties included in the model was 1.7 times greater than that for fulvic acid. The nitrogen content of SHHA is twice that of FA4; therefore, twice the concentration of amino acid moieties was considered (represented by MHBS). However, the best fit to the humic acid binding curves was obtained for a MHBS concentration equivalent to 50% of the assumed amino acid content. Model A was comprised of: citric acid, 1.224×10^{-5}; tricarballylic acid, 2.448×10^{-5}; salicylic acid, 1.224×10^{-4}; malic acid, 2.448×10^{-5}; acetylacetone, 2.72×10^{-5}; and MHBS, 8.0×10^{-6} mol L^{-1}. Model B was the same except the concentration of MHBS was 1.6×10^{-5} mol L^{-1}. The corresponding calculated binding curves are given in Figure 6.13. A lower concentration of acetylacetone resulted in a poorer fit to the data.

Although the models provide a reasonable fit to the humic acid data at a Cu(II):COOH ratio of 1:4.5, Figure 6.13 indicates that insufficient very strong Cu(II) complexors are included in the simulations.

Unlike fulvic acid, the models did not provide a very good fit to the humic acid data at the lower Cu(II) concentration; Figure 6.14. At this metal-to-ligand ratio the strongest binding sites would be utilized and it is possible that some moieties other than those considered here become important.

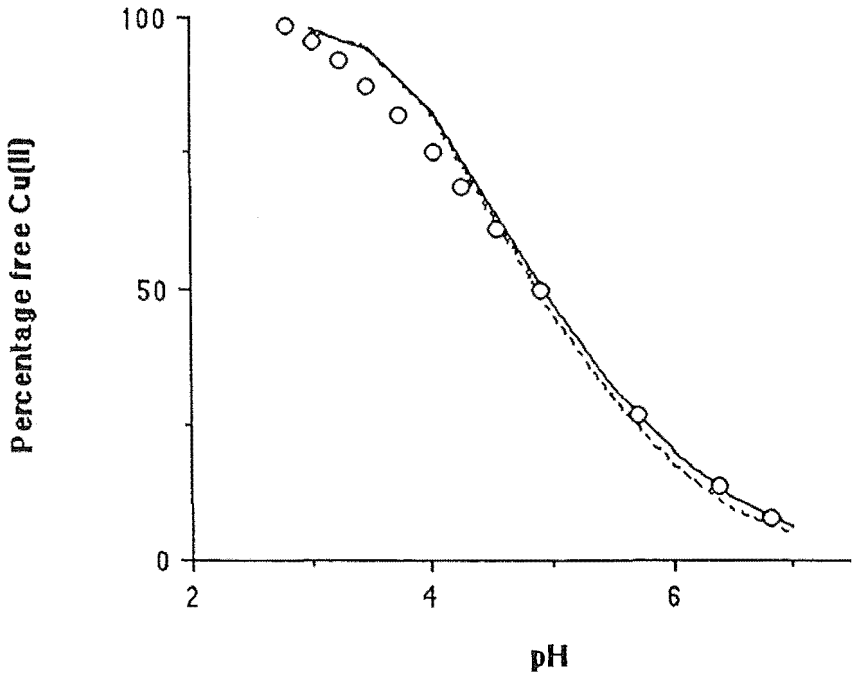


Figure 6.13: Cu(II) Binding Curves for Humic Acid and Mixtures of Model Ligands: [Cu(II)] = 4.0 x 10^{-5} mol L^{-1}

O SHHA, [COOH] = 1.8 x 10^{-4} mol L^{-1}; — model A; --- model B.

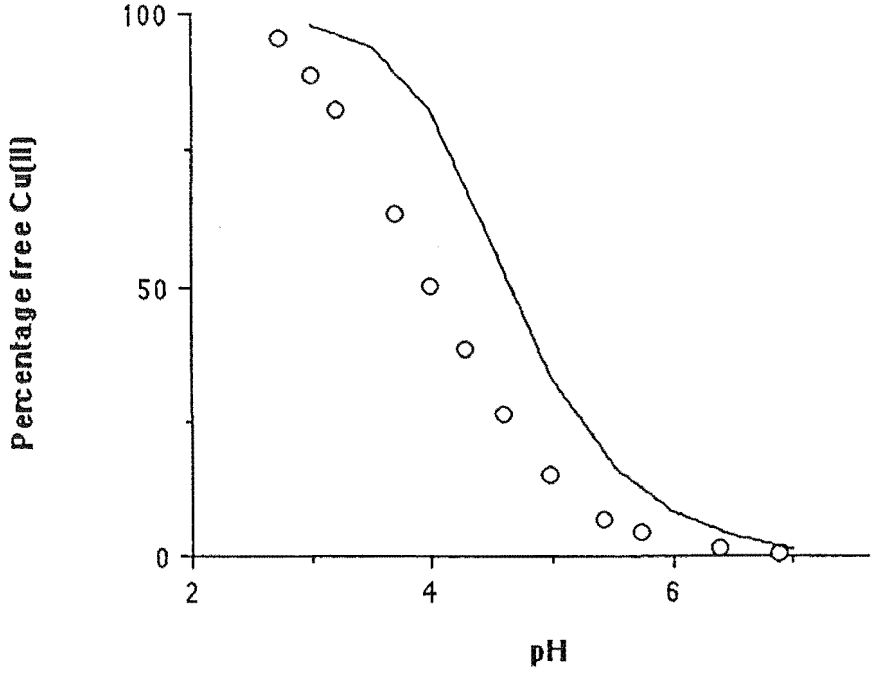


Figure 6.14: Cu(II) Binding Curves for Humic Acid and Mixtures of Model Ligands: [Cu(II)] = 9.0 x 10^{-6} mol L^{-1}

O SHHA, [COOH] = 1.8 x 10^{-4} mol L^{-1}; — Models A and B.

Ternary Complexes

Although formation of 1:2 metal-to-ligand complexes would be minimal at the concentrations involved, it is possible that ternary complexes contribute to Cu(II) binding. To investigate this, the binding curve for FA4 (1:4.5 ratio) was measured in the presence of salicylate at concentrations equal to 10% and 40% of the fulvic acid carboxyl group content. No change in the curves was observed. This result also indicates that fulvic acid moieties are stronger Cu(II) complexors than is salicylate.

The extent of formation of ternary complexes in the mixtures of discrete ligands was also calculated. A term was included in the simulation program for a ternary species (MAB) involving complexation between a metal ion (M) and two different ligands (A and B). This species had a statistically defined stability constant:
$\log K = 0.5(\log K_{MA_2} + \log K_{MB_2}) + 0.30$. No evidence for the formation of ternary complexes was obtained.

6.4.5 Effect of Competing Metal Ions on Cu(II) Binding Curves

Potassium

Although increasing the concentration of potassium (KNO_3) from 0.10 to 0.60 mol L^{-1} had no effect on the Cu(II) complexation capacity of fulvic acid, the metal binding curves for both humic and fulvic acids were displaced to higher pH (weaker binding); Figure 6.4.

The configuration of humic molecules may be altered at the higher ionic strength (Ghosh & Schnitzer, 1980) thus affecting their ability to bind Cu(II). Alternatively, the high concentration of potassium (10 000 to 60 000 times that of Cu(II)) may allow this ion to compete for Cu(II) complexation sites. To test this proposal, the Cu(II) binding curve for malonic acid was calculated for 0.10 and 0.60 mol L^{-1} K(I) ($\log K = 0.68$; Daniele et al., 1985b) (1.8×10^{-4} mol L^{-1} malonate, 9.0×10^{-6} mol L^{-1} Cu(II)). The curve for 0.60 mol L^{-1} K(I) was displaced to higher pH, by 0.075 at pH 4.0 and 0.825 at pH 4.75 indicating that potassium ions can compete for Cu(II) complexation sites under these experimental conditions.

Mg(II)

The Cu(II) binding curve for fulvic acid was displaced to higher pH in the presence of 0.06 mol L^{-1} Mg(II); Figure 6.4. For citric acid, calculations indicated that the amount of Cu(II) complexed was decreased by 46% in the pH range 4 - 6 in the presence of 0.06 mol L^{-1} Mg(II) (1.8×10^{-4} mol L^{-1} citrate, 9.0×10^{-6} mol L^{-1} Cu(II)). This indicates that Mg(II) can compete for Cu(II) complexation sites under these conditions.

Stability constants for the complexation of Ca(II) by carboxylate ligands are numerically similar to those for Mg(II). Other workers have reported minimal effects of Ca(II) (up to 0.01 mol L^{-1}) on the Cu(II) complexation capacity of humic acid (Hering & Morel, 1988a,b) or fulvic acid (McKnight & Wershaw, 1989). McKnight and Wershaw (1989) proposed that Ca(II) could compete only for a portion of the Cu(II) binding sites. Buffle et al. (1980) found that Ca(II) ($< 5 \times 10^{-3}$ mol L^{-1}) had a negligible effect on Cu(II)-humic binding at pH >6. Hering and Morel (1988b) suggested that either Ca(II) and Cu(II) do not compete for the same humic acid binding sites, or a binding mechanism other than complete complexation by discrete ligands is operative. The effect of divalent ions on the complexation of Cu(II) by humic substances is discussed further in Chapter 8.

Al(III)

The Cu(II) binding curve for FA4 (1:4.5 ratio) was measured in the presence of Al(III) (added as the nitrate salt); Figure 6.5. The curve was displaced to higher pH in the pH range 3.5 - 6.5 indicating competitive complexation by Al(III); a greater effect was observed for the higher concentration of Al(III). In contrast to Ca(II) or K(I), each curve became coincident with that in the absence of Al(III) at pH >6.5. At pH >6.5 Cu(II) may displace fulvic-bound Al(III), or both metal ions may be complexed by the humic substance.

Cavallaro and McBride (1980) reported suppression of the free Cu(II) concentration (ISE) in the presence of Al(III) in chloride media. However, in the present work (I = 0.10 mol L^{-1} KNO_3) no change in the EMF of a 1.0×10^{-5} mol L^{-1} Cu(II) solution was observed in the presence of 5.0×10^{-5} mol L^{-1} Al(III) at pH 2.5.

To investigate the coordination of Cu(II) in the presence of Al(III), Cu(II) binding curves were calculated (*via* the equilibrium program SIAS described in Chapter 8) for the

discrete ligands salicylate and malonate (1.8×10^{-4} mol L^{-1} ligand, 4.0×10^{-5} mol L^{-1} Cu(II), 4.0×10^{-5} mol L^{-1} Al(III)). (For malonic acid, Al(III) did cause displacement of the Cu(II) binding curve to higher pH, by 0.25 at pH 4.0 and 0.5 at pH 5.0.) The species distribution diagram indicated that in the pH region 3.5 - 6.0 Al(III) effectively competes with Cu(II) for coordination by malonate; at pH >6.0 $Al(OH)_4^-$ became the predominant Al(III) species, while most of the Cu(II) remained complexed with malonate (not shown). At a lower ligand concentration (2.7×10^{-5} mol L^{-1}), Cu(II)-malonate complexation was almost completely suppressed by Al(III), with significant formation occurring only above pH 6.0 (where Al(III) is hydrolyzed); Figure 6.15. That is, on the basis of this model ligand it is likely that Al(III) does not remain bound to humic substances above pH 6. Further, this indicates that Al(III) is bound more strongly by fulvic acid than is Cu(II) in the pH range 3.5 to 6.0.

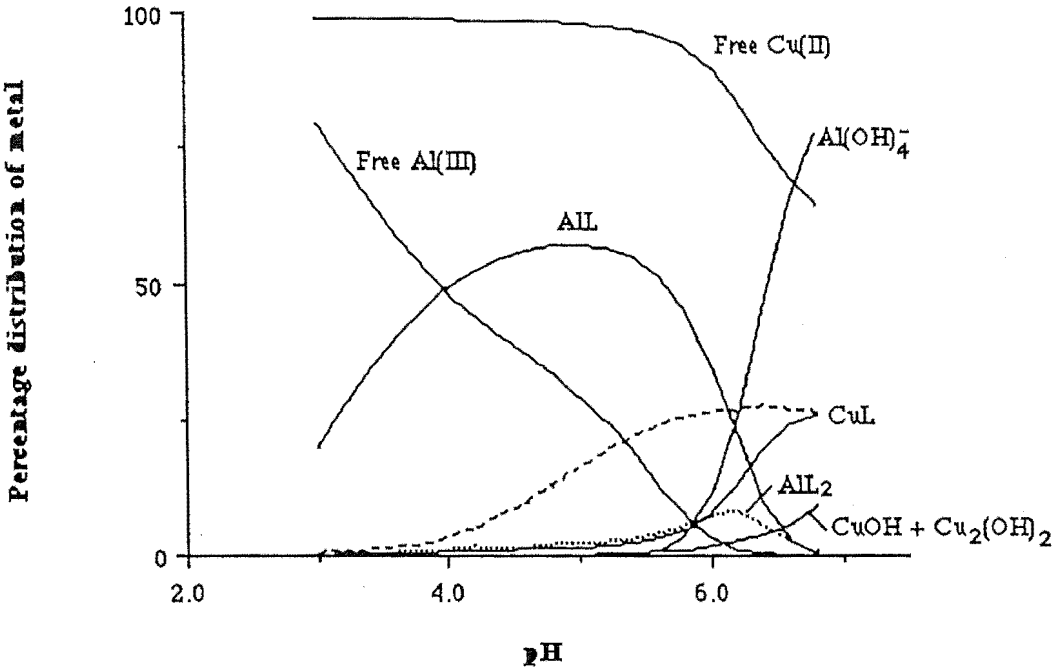


Figure 6.15: Species Distribution for Malonic Acid (2.7×10^{-5} M) in the Presence of Cu(II) and Al(III) (both 4.0×10^{-5} M)

Distribution of CuL in the absence of Al(III) (- - -).

Similar calculations for salicylate, indicated that Al(III) had a negligible impact on Cu(II) complexation. Al(III) was complexed significantly in the pH range 3.5 - 6.0 but had little effect because the Cu(II)-salicylate species is not very stable. Above pH 6

$Al(OH)_4^-$ was again the dominant Al(III) species. Therefore, it is probable that complexation of Al(III) with salicylate type moieties in humic substances would not alter Cu(II) binding. (It could, however, lower the affinity of an adjacent complexation site for Cu(II).)

Environmental Implications

These results have important implications for speciation of metals in the environment. Al(III) could have a significant impact on Cu(II) speciation in the pH range 3.5 - 5.5 typical of natural humic waters and Podzolic soils. Indeed, environmental levels of Al(III) are significantly greater than those of Cu(II). Typical concentrations of Al(III) are: 5×10^{-6} to 1×10^{-4} mol L^{-1} in soil solution and, 7.4×10^{-7} to 1.8×10^{-6} mol L^{-1} in acidic lake waters (Dr H.K.J. Powell, pers. comm., 1991). In contrast, the typical Cu(II) concentration in freshwaters is 4.7×10^{-8} mol L^{-1}; that in soil interstial waters is 1.5×10^{-7} to 9.5×10^{-7} mol L^{-1} (Buffle, 1988).

With regard to Cu(II) complexation by humic substances in seawater, pH 8.2 (Chapter 8) the ISE results indicate that strong complexation should occur in this medium. That is, humic substances may have sufficient capacity to coordinate both Cu(II) and other metal ions in solution, but may not compete strongly against metal hydrolysis. Measurement of metal ion complexation by humic substances in the pH range 6 to 9 is fraught with difficulties (Buffle, 1980).

The extensive hydrolysis chemistry of Al(III) explains why $Al(OH)_4^-$ became the major Al(III) species at pH >6, whereas ions such as K(I) and Mg(II) would remain complexed to the organic ligand. It is likely that Al(III) competes for Cu(II) complexation sites with different affinity than does Ca(II) or Mg(II). For example, Tam and McColl (1990) reported that at pH 4.5 the Al(III) binding affinity of α,β- substituents decreased in the order: COOH-OH > OH-OH > COOH-COOH > COOH; for Ca(II) the order was: COOH-COOH > COOH-OH > COOH > OH-OH.

The Cu(II) ISE appears to be a very useful and relatively simple probe for studying the complexation of humic substances with a range of metal ions.

6.4.6 Summary

On the basis of carboxyl group content, humic acid had a greater complexation capacity and a greater binding strength than did fulvic acid. This indicates that the fewer carboxyl groups in humic acid are distributed more 'efficiently' in terms of metal binding than those in fulvic acids (or in a mixture of discrete ligands at the same carboxyl group concentration). The humic acid carboxyl groups could also be in close proximity to other coordination sites which facilitate strong Cu(II) complexation.

The larger humic acid molecules exhibited both a greater complexation capacity and a greater binding strength than did the smaller molecules. This result indicates the possible importance of the larger molecular size humic fraction in environmental processes.

The presence of other weakly complexing metal ions, such as Mg(II), at relatively high concentrations inhibited Cu(II) complexation; Al(III) was preferentially bound in the pH range 3.5 - 5.5. Studies on the coordination of isolated humic substances to single metal ions cannot therefore be used directly to estimate the speciation of metal ions in the environment.. Further detailed studies on the competitive complexation of metal ions by humic substances (preferably performed *in situ*) will be important in understanding their role in determining the speciation of trace metals in soils and natural waters.

From comparison with mixtures of model ligands, the binding curves for both fulvic and humic acids could be modelled by simple carboxylate moieties (a greater effective concentration was required for humic acid). The types of moieties likely to dominate Cu(II) complexation by humic substances in weakly acidic and neutral solutions are citrate and malonate; salicylate and phthalate moieties are unlikely to be involved except in alkaline media. Our ability to simulate humic substance complexation is affected by a lack of detailed knowledge on the nature of humic substances themselves. Other types of functional groups such as dihydroxy species may participate in Cu(II) complexation. Inclusion of acetylacetone in the model greatly improved the fit to the humic substance data. The nitrogen content of humic substances is poorly characterized (Schnitzer, 1985; Chapter 1); improved understanding of this fraction may indicate the presence of other important Cu(II) chelating groups. Calculations with the model ligands glycylaspartic acid and MHBS indicated that such species (even when present in very low

concentration) have a significant impact on the Cu(II) binding curve. It is noted that the majority of amino acid moieties in humic substances (and in natural waters) are peptide-bound (Simonart et al., 1967; Brisbane et al., 1972; Biederbeck & Paul, 1973; Tuschall & Brezonik, 1980)

It is acknowledged that the approach used to simulate the humic substance binding curves is very simplistic. Nevertheless, it provides a useful basis for identifying the types of moieties which are most (and least) likely to be involved in Cu(II) complexation. Importantly, the results obtained suggest that metal ion coordination by both fulvic and humic acids involves predominantly aliphatic carboxylate moieties. Further, such an approach allows the researcher to gain an understanding of the effects of various parameters on metal complexation by a complex system (e.g. the effect of pH, ionic strength, metal-to-ligand ratio, presence of competing metal ions).

Any 'realistic' model of metal ion complexation by humic and fulvic acids must take into account the heterogeneity of these substances as a class of ligands, their ability to form species other than simple 1:1 complexes, and the presence of more than one phase. Some types of 'polyelectrolyte' effects may also be important. For example, the Cu(II) complex of polyacrylic acid is much more stable than that of the monomeric analog (Gregor et al., 1955). (It is noted that humic substances should not be regarded strictly as polyelectrolytes (Aiken & Malcolm, 1987; van den Hoop et al., 1990).) Further, postulating the existence of discrete binding sites in humic substances could be misleading. For example, Senesi (1986) observed that fulvic acid moieties responsible for Cu(II) complexation behaved as units integrated into a polymeric structure, and not as independent molecules.

CHAPTER 7

ANODIC STRIPPING VOLTAMMETRY. STUDIES ON THE APPARENT LABILITY OF Cu(II) AND Pb(II) COMPLEXES WITH HUMIC AND FULVIC ACIDS

7.1 INTRODUCTION

Anodic stripping voltammetry (ASV) is a very sensitive technique which has been applied to measurement of reducible metal ions in solution at environmentally significant concentrations. In this two-step technique metal ions are firstly concentrated from solution by reduction (at a constant potential for a fixed period of time, with stirring of the solution or rotation of the working electrode) and form an amalgam with the mercury electrode (the deposition step). An anodically ramping voltage is then applied and the metal is oxidized from the electrode giving rise to a current (the stripping step). Processes which affect the deposition and/or stripping steps will influence the measured current.

7.1.1 Electrode Systems for ASV

The electrodes which have been most frequently applied to trace metal analysis are the hanging mercury drop electrode (HMDE) and the thin-mercury film electrode (TMFE). The properties of these electrodes have been evaluated by Batley and Florence (1974). There are important differences between these electrodes. The surface-to-volume ratio for the TMFE is at least three orders of magnitude greater than that for the HMDE, resulting in much higher metal concentrations in the mercury film (Batley & Florence, 1974). The use of high frequency techniques, e.g. differential pulse (DP), at a HMDE greatly increases the peak current over that obtained with linear sweep voltammetry (DC). In contrast, at a TMFE similar peak currents are obtained for pulsed waveforms and for a rapid DC scan. DP-ASV at a HMDE has a limit of detection comparable to that at a TMFE. A distinct advantage of the TMFE is that the resolution is greater than that obtainable at a HMDE. However, to obtain the results of highest

precision at a TMFE the peak current for the second or third deposition-stripping cycle should be used (Batley & Florence, 1974).

A TMFE is much more susceptible to the formation of intermetallic phases than is an HMDE because of the higher concentration of metals resulting from deposition into the mercury film (Batley & Florence, 1974). In the presence of intermetallic compounds, the stripping peaks for the constituent metals may be depressed, the peak potential may be shifted, and multiple peaks may be observed. However, a TMFE is less prone to interferences from the adsorption of organic compounds on the electrode surface than is a HMDE (Batley & Florence, 1974; Wang & Luo, 1984).

There are other important differences between a HMDE and a TMFE. Firstly, the diffusion layer at a HMDE is spherical while that at a TMFE is linear. Further, a much thinner diffusion layer is attainable at a TMFE because the TMFE can be rotated at a much higher rate than the solution can be stirred for a HMDE. Secondly, a TMFE contains two very different surfaces on which metal may be deposited, namely the mercury droplets and the bare glassy carbon surface.

The HMDE is the electrode of choice for speciation studies because it allows the use of preconcentration techniques as well as being a fully renewable electrode. However, for speciation studies, a sound theoretical relationship between the flux of reducible species and the measured current must be established (Buffle, 1988). This is not possible at a HMDE because the hydrodynamic conditions around the drop during the deposition step are turbulent and ill-defined. Recently, a new electrochemical cell has been reported which enables a well-controlled, quasi-laminar flow to form around the HMDE. This results in a flux which is reproducible and predictable on theoretical grounds (Tercier et al., 1990; Tercier & Buffle, 1990).

7.1.2 Lability of Metal Species

ASV has been applied to studies of metal speciation in natural samples. Speciation of an element is defined as "the determination of the concentrations of the different physico-chemical forms of the element which together make up its total concentration in the sample" (Florence, 1986).

Lability is an important parameter in speciation analysis. The toxic, or bioavailable, fraction of a metal is that amount which can be transported across a membrane surface (Florence, 1986). Although the analogy may be an oversimplification (Nurnberg, 1983; Lund, 1986), a parallel has been drawn between the process of ASV electrodeposition and facilitated metal accumulation by organisms (Whitfield & Turner, 1979; Turner & Whitfield, 1980; Florence, 1986). The electrochemical and solution parameters can be chosen so that the ASV labile fraction of the total metal in solution is similar to the toxic fraction. That is, the degree of dissociation of a metal complex in the electrode diffusion layer and that at the surface of a biomembrane are comparable (Florence, 1984). However, the toxicity of lipid-soluble metal complexes cannot be predicted from their ASV lability (Florence et al., 1983; Turner, 1984).

Labile metal has been defined as that fraction of the total metal "that can be reduced at, and deposited into, a mercury electrode from stirred solution" (Florence, 1986). van Leeuwen et al. (1989a) considered labile species to be those complexes having such large "association"/dissociation rate constants that they do not limit the availability of oxidant in the diffusion layer. Labile metal is comprised of the free (aqua) metal ion and metal in complexes which dissociate during their lifetime in the diffusion layer. ASV is a dynamic technique which draws current through solution; therefore, the very act of measuring a system by ASV will disrupt ionic equilibria. This aspect was seen as an advantage by Buffle et al. (1976); voltammetric techniques were considered to be a good means of perturbing a system in order to study its properties.

Many controllable experimental factors affect the apparent lability of a metal ion, *viz:* deposition potential, rate of stirring of the solution or electrode rotation speed, the particular electrode used (e.g. HMDE or TMFE), wave form (e.g. DC or DP), pH, temperature, and buffer composition. Hence, in the analysis of natural samples "the measured concentration of ASV-labile metal can only be operationally defined by the

instrumental and solution conditions used and, in most instances, little information can be deduced about the electrode processes involved" (Florence, 1986).

In order to gain a meaningful estimate of the ASV labile fraction it is important to be aware of the potential problems associated with this technique and to compensate for these where possible. If ASV lability measurements are to be used as an estimate of the toxic fraction of a metal then the effects of any components on the stripping step must be eliminated or quantified. This allows the ASV-labile fraction to be equated to the "electroactive fraction" (the amount of metal deposited into the electrode during deposition) (Morrison et al., 1990). Potential interferences include: adsorption of organic matter on the electrode surface, tensammetric waves, and the presence of directly reducible complexes.

Tensammetric peaks arise at the adsorption and desorption potentials of surfactants on the electrode surface. They have no Faradaic component. In the potential range where the surfactant is adsorbed the base current is depressed markedly (Jacobsen & Lindseth, 1976). Interference from these adsorption waves (which may appear at potentials similar to those for Cd(II), Pb(II), or Cu(II)) can be overcome by use of a DC scan (Florence, 1986).

Directly reducible complexes, where electrons are added to the complex without its prior dissociation in the diffusion layer, can contribute to the apparent ASV labile metal, especially if very negative deposition potentials are used. The presence of such complexes can be detected by construction of pseudopolarograms (plots of stripping peak current *versus* deposition potential). In the absence of directly reducible complexes the peak current will increase from zero to a limiting value over a narrow range of deposition potential. The contribution of these species to the measured peak current can be minimized by use of a deposition potential which is just sufficiently negative to yield the maximum peak current for the free metal ion (Florence, 1986).

Metal Complexes With Macromolecular Ligands

The use of voltammetric techniques for the study of metal complexation by macromolecular ligands has recently been critically reviewed (van Leeuwen et al., 1989a). According to these authors, there are three problems associated with the interpretation of voltammetric studies on such systems. Firstly, the nature of the electrode process which controls the overall reduction rate must be determined (i.e. dissociation *versus* diffusion). Secondly, macromolecular ligands exhibit more complicated behaviour (such as polyelectrolytic, gel, or aggregation properties) than do simple systems. And thirdly, voltammetric measurements on heterogeneous natural macromolecules represent an average of the contributions of all moieties "weighted in an often complicated manner because of their different chemical equilibria, chemical kinetics and diffusion rate transport" (van Leeuwen et al., 1989a).

Because of their size, macromolecular metal complexes generally have a smaller diffusion coefficient than that of the free metal ion (Cleven et al., 1987). Erroneously, the resultant decrease in peak current on addition of these substances to a metal ion solution may be interpreted as the apparent nonlability of the complex. For a system containing labile macromolecular complexes, the simultaneous diffusion of labile species with different mobilities will be occurring. That is, the simultaneous diffusion of the metal complex (ML) and the free metal ion (M) towards the electrode surface results in a diffusion layer with an apparent thickness that is intermediate between that for pure ML and pure M (van Leeuwen, 1987). Under these conditions the reduced peak current is controlled by a mean diffusion coefficient (Cleven et al., 1986).

A further complication in interpreting voltammetric measurements on these metal-ligand systems is that the M:L ratio may vary greatly at the electrode surface over the course of the experiment. During the deposition step, reduction of ML will release free ligand at the electrode surface. This free ligand is then able to complex with inwardly diffusing metal ions (Stolzberg, 1977). The opposite effect is observed during the stripping step with the concentration of metal ions at the electrode surface being 30 to 300 times larger than that in the bulk solution (Buffle, 1981 ; Mota et al., 1985). In a complexing medium the shape, peak current, and peak potential of the ASV curves will be different from that for a ligand-free system, although at very long deposition times

(>20 min) the majority of M° may be oxidized to free M(II). It has been calculated that in order to avoid this effect of surface solution stoichiometry during the stripping step the ligand:metal ratio in the bulk solution must be greater than 1 000 (Mota et al., 1985).

Humic substances are heterogeneous and contain components which may adsorb on to the mercury surface or may complex metal ions, thus inducing the formation of adsorbed complexes (Buffle & Greter, 1979; van Leeuwen, 1984; Nelson, 1985a,b). Further, these adsorption and complexation properties may vary from one humic moiety to another resulting in a number of competition reactions which cannot be discriminated from one another owing to the complexity of the mixture (Buffle et al., 1987a). For such a system, a description in terms of overall parameters may be the most useful, provided that their conditions of applicability are clearly stated (Buffle & Cominoli, 1981).

7.1.3 Adsorption of Organic Substances on the Electrode Surface

The adsorption of organic material on the electrode surface is a frequent problem in analysis of natural samples. The adsorption of humic substances on a mercury electrode modifies the capacitive current due to a change in the double layer structure. In addition, the Faradaic current may be altered by the reduction or oxidation of some humic moieties; this may depend on the degree of electrode coverage and on the size of the humic molecules (Cominoli et al., 1980). Further, it has been suggested that the adsorption of fulvic acid on a mercury electrode alters the local pH at the solution-electrode interface (Wilson et al., 1980).

In the presence of adsorbed organic matter, metal stripping peaks in DC mode are often diminished and shifted in potential. It is difficult (perhaps impossible) to establish whether this decrease in peak current is caused by physical interference to diffusion and/or by formation of inert organic complexes. If the complexes formed are not completely inert then a decrease in peak current in the presence of organic matter could also be related to the rate of dissociation of the complexes. If the complexes are labile, the lower diffusion coefficient of the complex relative to the free metal could also decrease the peak current. When pulse techniques are used, adsorbed organic matter may also affect the kinetics of the stripping process, resulting in a broadening of the peak. In contrast to the effects noted above, adsorbed organic matter may also inhibit diffusion of

oxidation products away from the electrode surface, thus effecting an *enhancement* in the DP stripping peak current. Further, in the presence of adsorbed metal complexes (or ligand) the DP peak potential is a function of the amount of metal being oxidized from the electrode (Nelson & Mantoura, 1984).

Buffle et al. (1976) noted that ASV "offers the greatest number of possibilities of artefacts when chemical speciation is of interest". According to Florence (1986) "adsorption of surface-active substances (e.g. humic matter) from the sample solution on to the mercury electrode is one of the most serious complications in electrochemical speciation".

There has been extensive publication on the adsorption effects of organic substances on electrode surfaces. Virtually all studies have utilized the DP mode, and many refer to polarographic (as opposed to voltammetric) techniques. Differential pulse measurements are dependent on the rate of electron transfer reactions on the mercury surface and this wave form is particularly prone to adsorption interferences. Indeed, it has been reported that DP-ASV is not suitable for studying systems where the ligand may adsorb on the electrode surface (Gregor, 1987). Importantly, instrumental parameters may also be limiting with differential pulse. For example, Anson et al. (1976) reported that the PAR 174 potentiostat fails to discriminate completely against charging currents in the DP mode when capacitances as large as those produced by extensive reactant adsorption are introduced into the circuit. In contrast, with DC voltammetry the peak area obtained is independent of the rate of the stripping process. Recently, a comprehensive study on the individual effects of complexing agents and surfactants on the deposition and stripping steps in DP-ASV at a HMDE has been reported (Morrison et al., 1990). The results highlighted the limitations of DP-ASV for the determination of the toxic fraction of the total metal in solution.

It has been recommended that the determination of the lability of metal species in the presence of adsorbed ligands should be based on peak areas derived from DC-ASV measurements either at a HMDE (Gregor & Powell, 1988a) or at a TMFE (Powell & Florence, 1990).

In general, previous studies on the adsorption effects of a range of surfactants on the mercury electrode have reported that a decrease in ASV peak current is not always

observed, and that no general trend can be established (Sagberg & Lund, 1982). For example, Opperman et al. (1988) reported that with DP mode, humic acid suppressed Cu(II) peaks, but enhanced those for Pb(II) and Cd(II). Peaks were shifted cathodically in the presence of humic acid. The adsorption of surfactants was found to be pH and potential dependent; both the deposition and stripping steps were affected, probably in different ways (Opperman et al., 1988; Cleven et al., 1988). Indeed, Campanella (1987) stated that a particular adsorption effect exhibited by a surfactant is dependent on "chemical structure, concentration, environment, pH, metal, potential value, [and the] nature of the electrode". In some cases the adsorption characteristics of "simple" ligands have been studied in an attempt to model the behaviour of humic substances (e.g. Brezonik et al., 1976; Buffle et al., 1987a).

Various strategies have been employed to minimize or eliminate the adsorption effects of organic substances on the mercury electrode and to ensure that the ASV labile fraction depends only on the deposition step. The organic matter may be destroyed by ultraviolet irradiation (Batley & Farrar, 1978) or removed from the bulk solution by adsorption on fumed-silica (Kubiak & Wang, 1989a,b; Kubiak & Kowalski, 1989; Stauber & Florence, 1990). Medium exchange eliminates the effects of complexing agents and surfactants on the stripping step by replacing the sample solution with a simple electrolyte solution before the stripping step is commenced (Mann & Florence, 1987a). More recently, membrane-coated electrodes (Nafion and cellulose-acetate) have been applied to speciation analyses (Wang & Hutchins-Kumar, 1986; Morrison & Florence, 1989b).

7.1.4 Scope of This Work

In the present work, the adsorption effects of humic and fulvic acids on a range of electrodes (HMDE, TMFE, NCTMFE, and glassy carbon) were studied via DC-ASV (using peak area as a measure of charge). Humic substances were found to exert most of their effect on the deposition step. Results from this study allowed an equation for the ASV lability of humic-metal complexes to be established which compensates for the adsorption effects of humic substances and the pH dependence of the sensitivity of the

electrode systems and complex formation by humic moieties. No attempt was made to rigorously classify the nature of the complexes formed.

The adsorption characteristics of tannins (condensed and hydrolyzable) and Triton X-100 were also investigated. Tannins are heterogeneous and have been proposed as constituents of soil organic matter (Mindermann, 1979a,b,c). However, their adsorption characteristics on a mercury electrode were found to be quite different from those for humic substances. Triton X-100 is a nonionic surfactant which is commonly used in studies on the effects of surface-active species on the electrode response (e.g. Brezonik et al., 1976; Plavsic & Cosovic, 1989). The present work indicated that the adsorption of Triton X-100 on the mercury electrode has a much more dramatic effect on stripping peaks than do humic substances.

These studies also facilitated further understanding of the processes occurring at these electrode systems. It was concluded that no predictive generalizations can be made about the effects of surfactants on ASV measurements. The properties of each system must be studied under the conditions in which lability measurements are to be performed.

7.2 EXPERIMENTAL

All measurements were performed in a class 100 clean room and Milli-Q water was used to prepare all solutions. The working electrodes were used in combination with a laboratory-built platinum counter electrode and a Ag,AgCl reference electrode (in 1 mol L^{-1} KCl, with vycor junction). A PAR 174 potentiostat and a PAR RE0074 X-Y recorder were used.

All solutions were purged with oxygen-free nitrogen for 10 min prior to measurements. Unless otherwise stated a deposition potential of -0.9 V was used for Pb(II) measurements; -0.6 V was used for Cu(II).

Following the deposition step, the solution was left for 15 s, with no stirring, (the quiescent time) before the anodic scan was commenced. For measurements with a TMFE and a NCTMFE the electrode potential was held at 0.0 V for 30 s after each anodic scan to oxidize any residual metals in the mercury film.

7.2.1 HMDE

A PAR 303 static mercury drop electrode (Aristar mercury) was used. Solutions were stirred *via* a Teflon-coated magnetic stirrer bar using a PAR 305 stirrer and "fast" stirring rate (*ca.* 700 rpm). Measurements were performed in PAR glass cells (acid-washed, 1% Aristar HNO_3) containing 10 mL of solution. For DP-ASV a scan rate of 5 mV s^{-1} and a modulation amplitude of 25 mV were used; for DC-ASV the scan rate was 10 mV s^{-1}.

Between measurements the electrodes were stored in 1% Aristar HNO_3.

7.2.2 TMFE

Two glassy carbon electrodes were used. The "laboratory-built" electrode was constructed from a piece of glassy carbon (Tokai Chemical Co.), 3 mm diameter, (generously donated by Dr T.M. Florence) sealed in perspex. With this electrode measurements were conducted in laboratory-built perspex cells (designed to be accommodated in the PAR 305 stirrer) containing an inner glass sleeve with a sample volume of 5 mL. The solutions were stirred *via* a Teflon-coated magnetic stirrer bar; *ca.* 700 rpm.

The other electrode was a Metrohm 628-10 rotating disk electrode. The 6.1204.000 glassy carbon electrode had a disk diameter of 3.0 mm and was sealed in Teflon. Measurements with this electrode were performed in a laboratory-built perspex cell containing a sample volume of 35 mL.

For DC-ASV the scan rate was 100 mV s^{-1}; for DP-ASV a scan rate of 5 mV s^{-1} and a 25 mV modulation amplitude were used.

To prepare a thin-mercury film electrode, mercury was deposited at -1.0 V (*vs* Ag,AgCl) from a solution containing 7 x 10^{-5} mol L^{-1} mercury(II). With the laboratory-built electrode, mercury was deposited for 20 min (fast stir); a 10 min deposition time was used with the Metrohm electrode (electrode rotation speed, 2 000 rpm).

To prepare the Nafion-coated thin-mercury film electrode (NCTMFE), Nafion 125 (Aldrich), as supplied, was diluted with 'Spectroscopic' grade ethanol (BDH) to yield a solution containing 0.5% w/v Nafion. 1 µL of this solution was applied to the surface of a

glassy carbon electrode (*via* a microsyringe with a Teflon tip); the solvent was then evaporated with warm air from a hair dryer for approximately 60 s. Mercury was deposited through the Nafion film as described above for the formation of a TMFE.

The resulting TMFE or NCTMFE was then rinsed with Milli-Q water and transferred to the analyte solution. No attempt was made to avoid short term exposure of the electrode to oxygen (Tay et al., 1989; Powell & Florence, 1990; Wojciechowski & Bakerzak, 1990).

Electrode Maintenance

Initially, the glassy carbon electrodes were polished metallographically to a mirror-like finish (1 μm diamond paste). Thereafter, whenever a new electrode surface was prepared the electrode was cleaned by wiping the surface successively with Whatman 541 filter paper soaked in 'Spectroscopic' ethanol, then 1% Aristar HNO_3, then with wet and dry filter paper. The ethanol and HNO_3 remove any organic matter contamination, metal hydroxides, or calomel film which may have formed on the electrode surface (Florence, 1989). Electrodes maintained with this filter paper cleaning routine have retained their original calibrations and base current slopes for over five years (Florence, 1980). Between measurements the glassy carbon electrodes were stored dry in an empty cell.

7.2.3 Reagents

Measurements at pH 1.5 were performed in 0.03 mol L^{-1} Aristar HNO_3. Acetate buffer (1:1) was prepared from Analar acetic acid and Analar sodium acetate; an 0.01 mol L^{-1} solution was used to buffer samples to pH 4.8 (or 5.5, by addition of sodium acetate).

A stock mercuric ion solution was prepared by dissolution of Aristar mercury in Aristar HNO_3.

Triton X-100 (*iso*-octylphenoxypolyethoxyethanol) was obtained from BDH. Analysis of this surfactant, by ASV in acid solution, established that it did not contain any measurable reducible metallic impurities.

The Chinese Quince (condensed) tannin, an epicatechin polymer, (isolated from *Chaenomeles chinensis*) and the epicatechin dimer (B2) were obtained from Dr L.J. Porter. These tannins are referred to as the "condensed tannin" and the "B2-dimer" respectively. These samples have been described elsewhere (Kennedy & Powell, 1985; Powell & Rate, 1987). The hydrolyzable tannin (tannic acid) was Merck "pure". All tannin solutions were 0.025 μm membrane filtered before use.

The humic substances have been described in Chapter 3. Unless otherwise stated, all solutions were 0.025 μm membrane filtered before use.

7.2.4 Measurement of ASV Stripping Peaks

In this work, the peak area of the DC stripping wave was measured by cutting out and weighing the peak shape. The term "stripping peak" is used to denote this integrated current (which corresponds to the charge involved). For DP-ASV measurements, the height of the stripping wave was used as a measure of peak current.

7.2.5 Adsorption of Organic Substances on Electrode Surfaces

These experiments were performed at pH 1.5, 4.8 and 5.5. This method is designated as "procedure 1".

At pH 1.5, the ASV stripping peak (2 min deposition) was measured as a function of metal ion concentration (1×10^{-6} - 4×10^{-6} mol L^{-1}) in the presence and absence of organic matter (2 ppm). The percentage change in the stripping peak in the presence of organic compounds was calculated from the relative slopes for plots of Cu(II) concentration *versus* DC-ASV peak area.

At pH 4.8 and 5.5, complexation of Cu(II) by humic substances will occur, resulting in a decrease in the stripping peak. Therefore, in order to study the effect of adsorption of humic substances on the stripping peak, the experimental conditions must be chosen so that formation of metal-humic complexes is minimized. This was achieved in the present work by use of very low concentrations of humic substance (0.5 ppm) such that the complexation capacity was much less than the concentration of Cu(II) used in these experiments.

7.2.6 Apparent Lability of Metal-Humic Complexes

To measure the apparent lability of metal-humic complexes, the ASV stripping peak for a metal ion solution (1.2×10^{-7} mol L^{-1}) was measured, an aliquot of humic substance was then added (to generate a solution of *ca.* 1×10^{-3} mg mL^{-1}) and the measurement was repeated. This method is designated as "procedure 2". At least two measurements were made on each separate solution to ensure reproducibility of data. A 4 min deposition time was used. The equation used to calculate the apparent lability of metal-humic complexes from these data is given in Section 7.3.4. These concentrations of metal and humic substance were chosen to be consistent with previous work (Gregor & Powell, 1988a).

7.3 RESULTS

The amount of metal reduced after forced deposition from stirred solution (i.e. during the quiescent time and the time of the anodic scan) was subtracted from all measurements (Bond, 1980). This measurement is referred to as the "scan-only" peak.

7.3.1 General Characteristics of the Electrodes Studied

The electrodes used in this work (HMDE, TMFE, and NCTMFE) were compared in terms of their electrocapillary zero (EPZ), peak potentials, and ASV sensitivity.

Electrocapillary Zero (EPZ)

The presence of organic compounds had some effect on the EPZ (Table 7.1). For example, in the presence of humic substances at pH 4.8, the EPZ for a TMFE was -0.63 V, while that for a glassy carbon electrode was -0.62 V.

Table 7.1: Electrocapillary Zero Values

	EPZ (Volts)	
Electrode	pH 1.5[a]	pH 4.8[b]
HMDE	0	0, (-0.59[c])
TMFE	-0.35	-0.57
NCTMFE	-0.57	nd[d]
GLASSY CARBON	nd	-0.50

[a] 0.03 mol L^{-1} HNO_3.
[b] 0.01 mol L^{-1} acetate buffer.
[c] 0.1 mol L^{-1} KCl.
[d] not determined.

ASV Sensitivity

The TMFE and NCTMFE had similar DC-ASV sensitivity for Cu(II) and Pb(II) at pH 1.5 and 4.8. This has also been observed by Hoyer et al. (1987). The DC-ASV sensitivity of a HMDE was *ca.* 20 times lower than that obtained at a TMFE.

Peak Potentials

At a HMDE the Cu(II) DC-ASV peak occurred at -0.10 to -0.14 V at pH 1.5 and 4.8. At a TMFE the Cu(II) peak occurred at -0.05 to -0.08 V at both pH 1.5 and 4.8; at a NCTMFE the Cu(II) peak potential was in the range -0.11 to -0.15 V at pH 1.5 and 4.8. The Cu(II) DC-ASV peak at a bare glassy carbon electrode appeared at -0.05 V at pH 4.8. That is, the peak potential of the Cu(II) stripping peak was slightly variable. In contrast, the Pb(II) stripping peak occurred at *ca.* -0.50 V at all the electrodes studied (at pH 1.5 and 4.8).

The scan-only peak at a HMDE appeared at the same potential as a peak obtained following deposition. In contrast, the scan-only peak at a TMFE and a NCTMFE was 0.05 to 0.10 V more cathodic than that arising from controlled deposition.

7.3.2 Adsorption of Organic Substances on Electrode Surfaces

These measurements were made by use of procedure 1 (Section 7.2.5).

Hanging Mercury Drop Electrode (HMDE)

Humic Substances

The effect of adsorption of humic substances on the HMDE was measured at pH 1.5, 4.8 and 5.5 for copper(II) and lead(II) by DC-ASV. These effects on the Cu(II) and Pb(II) stripping peaks were measured separately from single metal ion solutions. Results are given in Table 7.2. All data refer to the percentage decrease in stripping peak when both deposition and stripping were performed in the presence of humic substances.

Table 7.2: Percentage Decrease in DC-ASV Peak Area in the Presence of Humic Substances[a] (HMDE)

	pH 1.5		pH 4.8		pH 5.5	
Sample	Cu(II)	Pb(II)	Cu(II)	Pb(II)	Cu(II)	Pb(II)
FA4	20	11	13	3	23	0
FA2	17	21	11	5	22	11
SHHA	7	24	9	0	10	nd

[a] relative to peak area in the absence of humic substances.

nd = not determined.

For comparison, some measurements were made using differential pulse mode; Table 7.3.

The presence of humic substances did not alter the peak positions at pH 1.5 or 4.8 when either DC or DP mode was used.

Table 7.3: Percentage Decrease in DP-ASV Peak Height in the Presence of Humic Substances (HMDE)

	pH 1.5		pH 4.8	
Sample	Cu(II)	Pb(II)	Cu(II)	Pb(II)
FA4	8	29	16	0
FA2	12	20	9	a
SHHA	0	a	a	0

a = reproducible stripping peaks could not be obtained.

Tannins and Triton X-100

In contrast to humic substances, tannins and Triton X-100 affected both the deposition and stripping steps at a HMDE (Tables 7.4 and 7.5). To assess the effect of these substances on the stripping step, Cu(II) and Pb(II) were deposited simultaneously from a ligand-free solution, then tannin or Triton X-100 was added in the last 10 s of deposition, followed by stripping into the ligand solution.

The sign of the adsorption effect (i.e. an increase or decrease in peak area) on stripping into ligand (following deposition in a ligand-free solution) was generally the same as that obtained for deposition and stripping in the presence of ligand. However, when the condensed tannin was present only in the stripping step it caused a *decrease* in the Cu(II) and Pb(II) stripping peaks at pH 4.8; in contrast, deposition and stripping in the presence of this tannin resulted in *increased* stripping peaks.

For these experiments, Cu(II) and Pb(II) were deposited simultaneously from a mixed solution using a deposition potential of -0.9 V.

In contrast to humic substances, the positions of the metal ion peaks were shifted when tannins or Triton X-100 were present during the deposition and stripping steps, or in the stripping step alone as shown in Table 7.6.

Table 7.4: Percentage Change in DC-ASV Peak Area for Stripping in the Presence of Tannins and Triton X-100 (HMDE)

(following deposition from a ligand-free solution)

	pH 1.5		pH 4.8	
Sample	Cu(II)	Pb(II)	Cu(II)	Pb(II)
Condensed tannin	-24	+16	-7	-10
B2-dimer	-21	+3	-22	-8
Tannic acid	-29	0	-24	-5
Triton X-100	+4	+14	-21	+24

Table 7.5: Percentage Change in DC-ASV Peak Area for Deposition and Stripping in the Presence of Tannins and Triton X-100; HMDE

	pH 1.5		pH 4.8	
Sample	Cu(II)	Pb(II)	Cu(II)	Pb(II)
Condensed tannin	-58	+10	+5	+8
B2-dimer	-26	+12	-30	-24
Tannic acid	-55	+13	-30	-27
Triton X-100	0	+8	-40	+15

Table 7.6: Shift in DC-ASV Peak Potential (Volts) in the Presence of Tannins and Triton X-100 (HMDE)

	pH 1.5				pH 4.8			
	Stripping		Deposition		Stripping		Deposition	
Sample	Cu(II)	Pb(II)	Cu(II)	Pb(II)	Cu(II)	Pb(II)	Cu(II)	Pb(II)
Condensed tannin	+0.01	0	+0.06	+0.005	0	0	0	0
B2-dimer	+0.025	0	+0.03	+0.005	0	0	0	0
Tannic acid	+0.025	0	+0.055	+0.02	0	0	a	-0.02
Triton	+0.015	+0.055	+0.02	+0.075	+0.01	+0.05	+0.015	+0.08

[a] a double peak was obtained.

Thin Mercury Film Electrode (TMFE)

Humic Substances

The effect of humic substances on the Cu(II) and Pb(II) stripping peaks was measured on separate solutions containing a single metal ion.

In contrast to observations at a HMDE, the copper(II) DC-ASV peak area measured at a TMFE was greatly enhanced in the presence of humic and fulvic acids at pH 1.5. When humic substances (FA4, FA2 or SHHA) were present in both the deposition and stripping steps, the copper(II) peak area was increased by a factor of 2 and the peak position was shifted by -0.02 V (relative to that in the absence of humic substance). For humic acid, this effect was the same at the laboratory-built electrode (using solution stirring) as at the Metrohm rotating electrode.

When humic acid was present only in the stripping step at pH 1.5, the Cu(II) DC-ASV peak area and peak position were not affected. At pH 4.8 humic and fulvic acids (when present in both the deposition and stripping steps) had no effect on the copper stripping peak, or peak potential, when either DC or DP mode was used.

With DP mode, the Cu(II) peak current at pH 1.5 was increased 8-fold on deposition and stripping in the presence of FA4 (the peak position was shifted by +0.04 V).

With Pb(II), the DC-ASV peak area was not affected by addition of humic or fulvic acids at pH 1.5 or 4.8.

Tannins and Triton X-100

The effect of tannins and Triton X-100 on the Cu(II) and Pb(II) stripping peaks measured at a TMFE at pH 1.5 and 4.8 is given in Table 7.7. For these experiments, Cu(II) and Pb(II) were deposited simultaneously from a mixed solution using a deposition potential of -0.9 V.

Tannins and Triton X-100 (0.004%) also had a significant effect when they were present only in the stripping step; Table 7.8.

Table 7.7: Percentage Change in DC-ASV Peak Area for Deposition and Stripping in the Presence of Tannins and Triton X-100 (TMFE)

		pH 1.5		pH 4.8	
Sample		Cu(II)	Pb(II)	Cu(II)	Pb(II)
Condensed tannin		-16[a]	-11[a]	+9	+17
B2-dimer		-20	-6	+13	0
Tannic acid		-20	+6	-11	0
Triton X-100:	0.004%	-68	-21	b	b
	0.008%	-65	-18	nd	nd
	0.016%	-35	0	nd	nd

[a] The effect of adsorption on these metal ions was determined individually.

[b] With successive deposition-stripping cycles, a continual decrease in the stripping peak was observed.

nd = not determined.

Table 7.8: Percentage Change in DC-ASV Peak Area on Stripping in the Presence of Tannins and Triton X-100 (TMFE)

	pH 1.5		pH 4.8	
Sample	Cu(II)	Pb(II)	Cu(II)	Pb(II)
Condensed tannin	-3	-3	+8	+9
B2-dimer	-9	0	-10	-2
Triton X-100	-14	-2	-13	-2

Nafion-Coated Thin Mercury Film Electrode (NCTMFE)

Humic Substances

At pH 1.5 and 4.8 the presence of FA4, SHHA, or unfiltered SHHA had no effect on the copper DC-ASV peak area. At pH 1.5, the Cu(II) peak position was shifted by -0.01 V in the presence of humic substances (increasing the concentration of humic substances, 0.5 - 3 ppm, had no further effect on the peak position); no shift in peak position occurred at pH 4.8.

With DP mode, FA4 had no effect on the Cu(II) peak current or peak position at pH 1.5.

Some adsorption experiments were also conducted at pH 7.5 using thallium(I) as a probe. Thallium will be complexed only very weakly by humic substances. For deposition from a 1.2×10^{-7} mol L^{-1} Tl(I) solution in 0.1 mol L^{-1} HEPES buffer, the presence of 50 ppm SHHA had no effect on the DC-ASV peak area as measured at a NCTMFE or a TMFE; whereas at a TMFE the peak potential was shifted by -0.05 V.

Tannins and Triton X-100

At pH 1.5, the condensed tannin and the epicatechin-dimer had no effect on the Cu(II) and Pb(II) stripping peaks. At pH 4.8, the tannin caused a 10% reduction in the Cu(II) DC-ASV stripping peak, and a 7% decrease in the Cu(II) DP-ASV peak current, but had no effect on the Pb(II) peak.

In contrast to humic substances and tannins, Triton X-100 did have a significant effect on the stripping peaks measured at a NCTMFE; Table 7.9. The DC-ASV Cu(II) peak potential was shifted by +0.05 V in the presence of Triton X-100 at pH 1.5 and 4.8; a +0.03 V shift occurred for Pb(II). With DP mode at pH 4.8, the Cu(II) and Pb(II) peaks were shifted by +0.03 V and -0.01 V respectively, on deposition and stripping in the presence of Triton X-100. For these experiments, Cu(II) and Pb(II) were deposited simultaneously from a mixed solution using a deposition potential of -0.9 V.

Table 7.9: Percentage Change in DC-ASV Peak Area on Deposition and Stripping in the Presence of Triton X-100 (NCTMFE)

	Cu(II)	Pb(II)
pH 1.5 0.004%[a]	+19	+130
pH 4.8 0.004%	+24	+16
0.008%	0	+44
DP[b]	-35[b]	-88[b]

[a]wt % Triton X-100.

[b]DP mode; the same result was obtained at 0.004 and 0.008% Triton X-100.

When Triton X-100 (0.004 wt %) was present only in the stripping step at pH 1.5, the Cu(II) and Pb(II) DC-ASV stripping peaks were increased by 4% and 13% respectively (and the peak positions were shifted by +0.015 V and +0.005 V respectively); no effect on peak area or peak potential was observed at pH 4.8.

7.3.3 Pseudopolarograms

The possible presence of directly reducible complexes was investigated by constructing pseudopolarograms for Cu(II) in the presence and absence of humic substances. For both fulvic and humic acid at a HMDE and a TMFE, the DC-ASV peak area increased from zero to a limiting value over a narrow range of deposition potential, indicating that the copper(II)-humic solutions did not contain directly reducible complexes.

7.3.4 Apparent Lability of Metal-Humic Complexes

These measurement were made by use of procedure 2 (Section 7.2.6). The fraction of apparent nonlabile complexes (α), at a given pH, was calculated from the equation:

$$\alpha = 1 - \frac{\text{pk area in presence of HS}}{\text{pk area in absence of HS}}$$

where the peak area in the presence of humic substance was corrected for the effects of humic substance adsorption on the particular electrode surface (*vide supra*). An example of this calculation is given in Section 7.4.6.

Hanging Mercury Drop Electrode

The apparent labilities of copper(II) and lead(II) complexes with humic and fulvic acids at a HMDE are given in Table 7.10. Each number pair in the table represents duplicate experiments (with separate solutions) with deposition from single metal ion solutions.

Table 7.10: Percentage Nonlabile Humic Complexes as Measured at a HMDE

	Cu(II)		Pb(II)	
Sample	pH 4.8	pH 5.5	pH 4.8	pH 5.5
FA4	65, 68	75	46	52
FA2	74	63, 50	41	50
SHHA	61, 61	75	38	52

Thin Mercury Film Electrode

The apparent lability of copper(II)-humic complexes at a TMFE at pH 4.8 and 5.5 is given in Table 7.11. For these measurements the solution was stirred at *ca.* 700 rpm and the laboratory-built glassy carbon electrode was used. Each number pair in the table represents duplicate experiments (with a freshly prepared TMFE and separate solutions).

Table 7.11: Percentage Nonlabile Copper(II)-Humic Complexes as Measured at a Laboratory-Built TMFE

Sample	pH 4.8	pH 5.5
FA4	10, 10	28
FA2	8, 11	29
FAS	14, 12	17, 23
SHHA	6, 5	34, 27

The lability of copper(II)-humic complexes at a TMFE was also studied as a function of electrode rotation speed (using the Metrohm rotating glassy carbon electrode); Table 7.12. Each row in the table corresponds to one experiment (with a fresh electrode surface and separate solutions).

Table 7.12: Percentage Nonlabile Copper(II)-Humic Complexes as Measured at a Rotating TMFE at pH 4.8

	Electrode Rotation Speed (rpm)					
Sample	500	1000	1500	2000	2500	3000
FA4	39	31	32	33	35	32
		30		30		25
		35		22		31
SHHA	22	16	19	21	25	24
		24		21		15

Parallel experiments were performed for Pb(II)- humic acid complexes; Table 7.13. Two separate experiments were performed.

Table 7.13: Percentage Nonlabile Pb(II)-Humic Acid Complexes as Measured at a Rotating TMFE pH 4.8

Rotation Speed (rpm)	% nonlabile complexes
1 000	9, 11
2 000	13, 16
3 000	20, 14

Nafion-Coated Thin Mercury Film Electrode

Lability measurements performed with the laboratory-built electrode (with stirring of the solution) were not reproducible; Table 7.14. Each number in the table represents one experiment (with a fresh electrode surface and separate solutions).

Table 7.14: Percentage Nonlabile Copper(II)-Humic Complexes as Measured at a Laboratory-Built NCTMFE

Sample	pH 4.8	pH 5.5
FA4	38, 9, 18, 34	39
FA2	25, 16, 27	31
FAS	36, 26	46
SHHA	38, 25	31
unfilt. SHHA	40, 23	28, 38

Use of a Nafion-coated thin-mercury film formed on the rotating electrode allowed much more reproducible data to be obtained; Table 7.15. Each row in the table represents one experiment (with a fresh electrode surface and separate solutions).

Table 7.15: Percentage Nonlabile Copper(II)-Humic Complexes as Measured at a Rotating NCTMFE, pH 4.8.

	Electrode Rotation Speed (rpm)					
Sample	500	1000	1500	2000	2500	3000
FA4		50		48		42
		46		47		44
SHHA	43	38	36	35	33	36
		41		34		31

The lability of copper(II)-humic acid complexes as a function of rotation speed was also measured at pH 5.5 at a TMFE and a NCTMFE; Table 7.16.

Table 7.16: Percentage Nonlabile Copper(II)-Humic Acid Complexes as Measured at a Rotating Electrode, pH 5.5

	Electrode Rotation Speed (rpm)		
Electrode	1000	2000	3000
TMFE	49	42	43
NCTMFE	43	43	42

Levich plots (i_p versus $w^{1/2}$) for data at the NCTMFE and TMFE (at pH 4.8 and 1.5) in the presence and absence of humic substances were linear ($r = 0.999$) and passed through the origin .

Effect of Fractionation of Humic Substances

Reverse-Phase Chromatography

The change in lability of copper(II)-FA4 complexes after components of the fulvic acid had been adsorbed on a Sep-pak C_{18} cartridge was measured (procedure 2). Approximately 50% of the coloured components of fulvic acid were adsorbed on the C_{18} cartridge; adsorbed components were quantitatively recovered by elution with methanol. At a HMDE, the FA4 which passed through a C_{18} cartridge formed copper(II) complexes which were 54% nonlabile at pH 4.8 (average of duplicate experiments; 53% and 55%); c.f. 68% nonlabile copper(II) complexes formed by the whole fulvic acid sample (Table 7.10). Further, these fulvic acid components had no adsorption effect on the copper(II) stripping peak at pH 1.5 or 4.8 (procedure 1). A parallel experiment with humic acid could not be performed because humic acid was quantitatively adsorbed on the C_{18} cartridge.

In contrast, measurements at a TMFE indicated that the fulvic acid filtrate formed approximately the same proportion of nonlabile copper(II) complexes as did the whole fulvic acid sample; *viz* 10%.

Centricon Molecular Size Fractionation

A fulvic acid sample was placed in a 10 000 molecular weight cut-off (MWCO) Centricon filter and centrifuged at 2 500 rpm for 20 min. At a HMDE, copper(II) complexes with the whole, unfractionated fulvic acid sample were 24% nonlabile at pH 4.8 (a different fulvic acid sample from those reported in Table 7.10 was used). The filtrate formed copper(II) complexes which were 11% nonlabile, while complexes with the retentate were 32% nonlabile.

SHHA components which passed through a 30 000 MWCO filter formed Cu(II) complexes which were 41% nonlabile, compared with 62% for the whole sample.

7.4 DISCUSSION

7.4.1 Electrocapillary Zero

The EPZ is the potential at which the net charge on the electrode is zero. The particular applied potential at which this occurs will depend on the ions present in solution. The specific adsorption of anions on the electrode causes a negative shift in the EPZ (Golub et al., 1989). Chloride, bromide, and iodide are specifically adsorbed on mercury; this explains the large negative shift in the EPZ for a HMDE in 0.1 mol L^{-1} KCl media (Table 7.1).

7.4.2 Choice of Ionic Strength

For DC-ASV the concentration of supporting electrolyte is not crucial. Florence (1970) reported that for Pb(II) determinations with a TMFE, the DP-ASV peak current and peak potential were not altered when the concentration of KNO_3 was varied from 0.005 to 1 mol L^{-1}.

In the present work, the DC-ASV stripping peaks obtained for copper(II) at a HMDE were the same in 0.01 and 0.1 mol L^{-1} KNO_3. With a TMFE and a NCTMFE a supporting electrolyte concentration of 0.1 mol L^{-1} (KNO_3) was used; this minimized the large scan-only peak which was otherwise obtained at these electrodes.

7.4.3 Peak Potentials

The Cu(II) DC-ASV peak occurred at a more positive potential at a TMFE than at a HMDE, indicating that it is more difficult to oxidize Cu(II) from the surface of a TMFE. Similarly, Cu(II) was more difficult to oxidize from a bare glassy carbon surface than from a mercury drop. However, the scan-only peaks at a TMFE were at more negative potentials than those obtained following a deposition step.

In explaining these observations, one must consider the two different surfaces on which metal may be deposited at a TMFE. The deposition and stripping of monolayers of metal on bare glassy carbon electrodes has been reported. However, such monolayer peaks appear at potentials which are more *positive* than that corresponding to a multilayer deposit; this indicates a stronger interaction between the metal

and the glassy carbon than the normal bonding of the metal with itself (Vassos & Mark, 1967; Eisner & Mark, 1970; Faulkner, 1984).

It is possible that the scan-only peak at a TMFE corresponds to Cu(II) being stripped from only the glassy carbon sites, or only the mercury sites, whereas the peak obtained following a deposition step represents some combination of stripping from these two surfaces.

The ASV stripping peak was measured as a function of Cu(II) concentration at a TMFE and a bare glassy carbon electrode (in the absence of ligands). At the bare glassy carbon electrode a single stripping peak was obtained (E_p = +0.09 V) and there was a linear relationship between the stripping peak and the concentration of Cu(II) (1.0×10^{-7} to 3.8×10^{-7} mol L^{-1}). At a TMFE this linear relationship also applied ([Cu(II)] = 5×10^{-8} to 2.2×10^{-6} mol L^{-1}); however, two stripping peaks were discernible at low concentrations of Cu(II). This was more pronounced with DP (Figure 7.1) than with DC mode (Figure 7.2). For DC-ASV measurements the total area under the two peaks was taken as a measure of the stripping charge. As the concentration of Cu(II) increased, a single stripping peak was observed. The smaller peak occurring at the more positive potential at low Cu(II) concentrations could arise from Cu(II) being stripped from the glassy carbon sites (*viz:* E_p for Cu(II) oxidation from bare glassy carbon was +0.09 V, c.f. E_p from a TMFE was *ca.* -0.05 V). Due to the very low solubility of Cu(II) in mercury (0.0006%; Stojek et al., 1976), a greater proportion of the stripping current may be expected to arise from metal deposited on glassy carbon (rather than on mercury) as the concentration of Cu(II) is increased. This may explain why the peak potential shifted to more positive values as the concentration of Cu(II) was increased. With DC-ASV the Cu(II) peak potential shifted from -0.085 V at 5.0×10^{-8} mol L^{-1} Cu(II) to -0.015 V at 2.2×10^{-6} mol L^{-1} Cu(II); with DP-ASV the peak potential shifted from -0.16 to -0.07 V over the same range of Cu(II) concentration. In support of this hypothesis, the peak potential for Cu(II) measured at a bare glassy carbon electrode (pH 4.8) was constant in the concentration range 1.0×10^{-7} to 3.8×10^{-7} mol L^{-1} (E_p = +0.09 V).

Alternatively, the observed anodic shift in the Cu(II) peak potential at a TMFE, with increasing Cu(II) concentration, could represent the formation of multiple Cu(II)-mercury phases (i.e. a heterogeneous amalgam). Indeed, Stojek et al. (1976) reported that the deposition of solid metals in mercury shifts the stripping peak anodically.

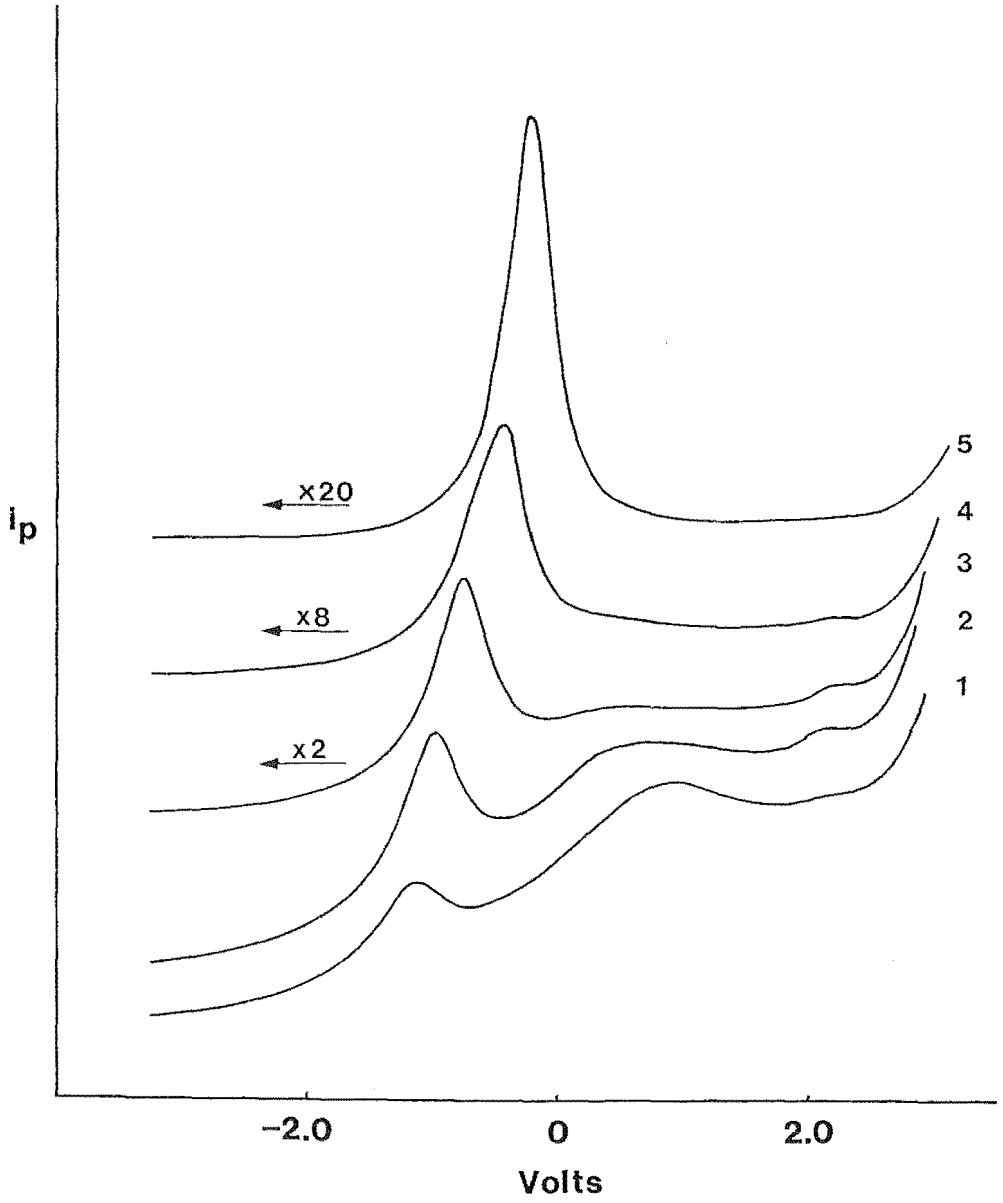


Figure 7.1: DP-ASV Stripping Peaks for Cu(II) at a Laboratory-Built TMFE, pH 1.7
[Cu(II)] = 5.0×10^{-8} M, (1); 1.0×10^{-7} M, (2); 2.5×10^{-7} M, (3); 9.9×10^{-7} M, (4); 2.2×10^{-6} M, (5).
Scan rate, 5 mV s^{-1}; 'fast' stir, 700 rpm;
Deposition potential, -0.9 V.

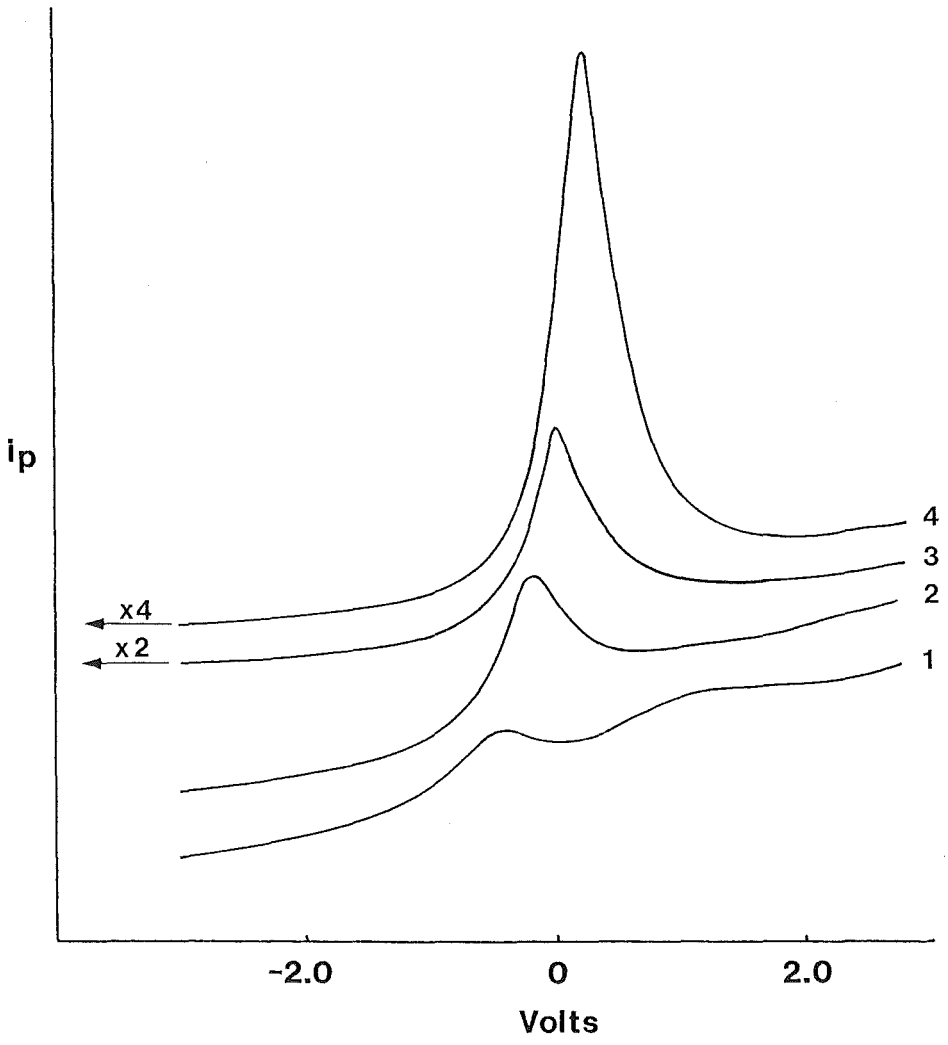


Figure 7.2: DC-ASV Stripping Peaks for Cu(II) at a Laboratory-Built TMFE, pH 1.7
[Cu(II)] = 5.0 x 10^{-8} M, (1); 1.0 x 10^{-7} M (2); 2.5 x 10^{-7} M (3); 9.9 x 10^{-7} M (4).
Scan rate, 100 mV s^{-1}; 'fast' stir; 700 rpm;
Deposition potential, -0.9 V.

Another possibility is that horizontal diffusion occurs in the mercury film during the anodic scan, especially at higher Cu(II) concentrations. That is, metal oxidized near the edges may be replaced by metal from the thicker centre of the film (or, more correctly, the centre of the mercury microdroplets). This process would require oxidation of metals from the thicker part of the film to occur at potentials more positive than would occur from the thinner part, resulting in a shift of the stripping peak to more positive values (Kounaves & Buffle, 1988).

According to Schönberger and Pickering (1980), the peak potential for a metal ion at a TMFE is dependent on the thickness of the mercury film, the rate of diffusion of oxidized species, the diffusion layer thickness, and the scan rate. Hence, it is probably not valid to compare the ASV peak potentials obtained at a HMDE with those measured at a TMFE.

7.4.4 Adsorption of Organic Substances on Electrode Surfaces

Hanging Mercury Drop Electrode (HMDE)

Humic Substances

The DC-ASV and DP-ASV stripping peaks measured at a HMDE were diminished in the presence of humic substances (procedure 1); Tables 7.2 and 7.3. The percentage decrease in peak area was greater for Cu(II) than for Pb(II). For Cu(II), the effect of humic substances was independent of pH over the range pH 1.5 to 4.8. In contrast, the Pb(II) peak was reduced to a greater extent at pH 1.5 than at pH 4.8. The use of low concentrations of humic substances in these adsorption studies ensured that metal-humic complexing did not contribute significantly to the stripping peak at pH >1.5 (Section 7.2.5).

In attempting to understand this, two perspectives were considered. Firstly, Cu(II) was deposited at -0.6 V, whereas Pb(II) was deposited at -0.9 V. The mercury electrode has been considered as a nonpolar hydrophobic surface whose surface potential can be adjusted independently of the pH of the solution. Nonpolar molecules would be adsorbed most strongly on the mercury electrode when the surface charge is near zero. These molecules will be replaced by water or electrolyte ions when the charge on the mercury surface is highly positive or negative (Ulrich et al., 1988). Although the electrocapillary zero (EPZ) of a HMDE in chloride media (at neutral pH) is approximately -0.6 V (versus Ag, AgCl), the EPZ in

acetate buffer (pH 4.8) and dilute HNO_3 (pH 1.5) media was found to be approximately 0 V. Hence the different deposition potentials used for Cu(II) and Pb(II) should *not* affect the adsorption of humic substances on the HMDE. Indeed, fulvic acid had the same effect on the Cu(II) stripping peak at pH 1.5 when deposition was carried out at -0.6 V and -0.9 V. It has been reported that fulvic acid adsorbs on a mercury electrode over a wide potential range, but adsorption is strongest at potential values close to or less negative than the EPZ with only weak adsorption occurring at potentials more negative than -0.9 V (Buffle et al., 1987b). According to Raspor and Valenta (1988), aromatic humic moieties will be adsorbed on a positively charged mercury surface; positively charged amino groups will be adsorbed at negative potentials.

Secondly, even though conditions were chosen to minimize any contribution to the decrease in stripping peak from complexation of the metal ions by humic substances, the relative affinity of humic substances for these metal ions may be important. Humic substances have a greater affinity for Cu(II) than for Pb(II) (Saar & Weber, 1982); further, as the pH increases so will the amount of metal complexation. However, the effect of adsorption of humic substances on the Cu(II) stripping peak (procedure 1) did *not* increase with pH, thus indicating that metal-humic complexing is not contributing significantly to these measurements.

The magnitude of the stripping peak measured by ASV is affected by factors which contribute to the deposition and/or stripping steps. It has been established that surfactants have an impact mainly on the deposition step in ASV, and the stripping step is usually not affected (Lukaszewski et al., 1979; Gregor & Powell, 1988a). A recent DC-ASV study of metal-fulvic acid systems found that fulvic acid has a negligible effect on the stripping process at a HMDE (Gregor & Powell, 1988a). This was also observed in the present work (for both humic and fulvic acid).

Effect of Reverse-Phase Chromatographic Fractionation of Humic Substances

Mercury provides a hydrophobic surface (Ulrich et al., 1988). Therefore, it was anticipated that the nonpolar humic moieties would be responsible for the decrease in stripping peaks observed at a HMDE in the presence of humics. This hypothesis was verified by passing a solution of FA4 through a Sep-pak C_{18} cartridge to remove the more "hydrophobic"

components. By use of procedure 1 it was established that the material which was not retained on the cartridge did not decrease the copper(II) peak area at pH 1.5 or 4.8.

Consistent with this observation for fulvic acid, experiments reported by Hunter and Lee (1986) indicated that humic acid also adsorbs on mercury via hydrophobic interactions. Humic acid, isolated from river water by hydrophobic adsorption onto Amberlite XAD-2 resin, was found to be almost 4 times more effective at suppression of streaming maxima than was the whole water sample (Hunter & Lee, 1986). Further, adsorption effects of surface active substances are more serious in ASV than in polarography because the stationary HMDE allows slow adsorption processes to reach equilibrium (Brezonik et al., 1976).

A study on the adsorption properties of fulvic acids at a mercury electrode, and compounds which were proposed as models for fulvic components, indicated that the hydrophobic part of the ligand had the greatest tendency to accumulate on the mercury surface (Buffle et al., 1987a).

Effect of Ionic Strength

Procedure 1 was used to investigate the influence of ionic strength on the effect of FA4 on the Cu(II) stripping peak at pH 1.5. In 0.1 mol L^{-1} KNO_3 medium, FA4 caused a 12% decrease in the Cu(II) peak area; no effect was observed in 0.6 mol L^{-1} KNO_3. Hence, the adsorption effects of fulvic acid become less significant as the ionic strength is increased. Fulvic acid is not expected to have a significant complexing capacity for Cu(II) at pH 1.5; therefore, this effect should not be due to a reduction in the complexing capacity of fulvic acid as the ionic strength is increased. The ionic strength dependence of fulvic acid adsorption on a mercury surface has also been reported by Cominoli et al. (1980).

Tannins and Triton X-100

Tables 7.4 and 7.5 indicate that these substances had a much greater impact on Cu(II) and Pb(II) stripping peaks at a HMDE than did humic substances. Other authors have reported that Triton X-100 has significantly different adsorption characteristics on a HMDE than do humic substances (Cosovic & Vojvodic, 1987).

Tannins and Triton X-100 had a significant effect on the stripping step (following deposition from a ligand-free solution); Table 7.4. In some cases this effect was almost as

large as that observed when both deposition and stripping were performed in the presence of the surfactant. A decrease in peak area on stripping in the presence of surfactant may arise if the surfactant inhibits movement of oxidized metal away from the surface of the mercury drop, resulting in a very broad stripping wave (which makes baseline determination difficult). It is difficult to explain how an increase in peak area could occur, as observed for Pb(II) at pH 1.5 in the presence of tannins and Triton X-100. With DP-ASV, metal ions may be redeposited into the electrode during the reverse pulse, then oxidized again, thus enhancing the current. Any substance which is adsorbed on the electrode may concentrate metal ions in the vicinity of the drop and thus enhance the current erroneously. However, in the present work a non-pulse mode of ASV was used, hence this mechanism cannot be in effect.

With humic substances, any adsorption effect (procedure 1) was only observed on deposition in the presence of ligand and was manifested in a decrease in the stripping peak. In contrast, tannins and Triton X-100 affected both deposition and stripping and generally caused a decrease in Cu(II) peak area and an increase in Pb(II) peak area. A decrease in the stripping peak following deposition from a ligand solution may arise if nonlabile complexes or complexes with a low diffusion coefficient are formed, and/or adsorption of the ligand on the electrode surface inhibits metal deposition into the mercury drop. It is not known how an increase in the stripping peak could arise.

In contrast to what was observed for humic substances, the effect of the condensed tannin on the Cu(II) DC-ASV peak area at pH 1.5 was dependent on the deposition potential used. With a deposition potential of -0.9 V the condensed tannin decreased the Cu(II) stripping peak by 71%; at -0.6 V the decrease was 58%.

Tannins and Triton X-100 also had a dramatic effect on the Cu(II) and Pb(II) peak potentials, E_p, causing shifts of up to +0.075 V in peak position (Table 7.6). A positive shift in peak potential relative to that in the absence of ligand indicates that it was more difficult to oxidize the metal from the mercury drop in the presence of these substances. Further, in the presence of the B2-dimer and tannic acid at pH 4.8, a split Cu(II) wave was observed (one peak occurred at the same potential as that for Cu(II) in the absence of tannin, and the other was 0.09 V more negative). This may represent two different Cu(II) oxidation products, *viz:* Cu-amalgam being oxidized to the free aqua metal ion, and to a Cu-tannin complex. For the latter case, the reaction will be limited by the concentration of tannin on, or near, the mercury

drop. For purposes of calculating the effect of these tannins on the Cu(II) stripping peak at pH 4.8 it was assumed that the total area of the double peak corresponded to Cu(II) oxidation.

The inherent rate of adsorption on mercury from aqueous solution is usually a rapid, mass transfer controlled process (Delahay, 1965). The adsorption of surfactants on a mercury electrode is expected to be rapid. Complete coverage of the electrode surface is established within fractions of a second at a surfactant concentration of 10^{-2}% (Lukaszewski et al., 1979). However, the adsorption of Triton X-100 on the mercury drop appeared to be slow and time dependent. When a drop of mercury was dispensed and left in stirred solution (with no applied potential) for 15 min before deposition was commenced the resultant Cu(II) stripping wave was shifted by +0.041 V from that for Cu(II) in the absence of Triton X-100; Pb(II) was shifted by +0.11 V (c.f. Table 7.6). The Cu(II) and Pb(II) stripping peaks were also decreased by approximately 10% (c.f. Table 7.5). Replicate depositions on fresh mercury drops (with no waiting time before commencement of the deposition step) resulted in reproducible stripping peaks and peak potentials (Tables 7.5 and 7.6). Hence, it was inferred that no changes in the bulk solution chemistry were occurring over time. A parallel effect was not observed for humic substances or tannins.

It is possible that Triton X-100 permeates the surface of the mercury drop and thus alters the nature of the metal amalgam. Alternatively, the structure of the adsorbed layer of Triton X-100 on the mercury surface may change over time. For example, multilayer adsorption and micelle formation in the adsorbed layer can occur especially with large adsorbed molecules (Delahay, 1965).

Thin Mercury Film Electrode (TMFE)

Humic Substances

In contrast to what was observed at a HMDE (Table 7.2), the presence of humic substances caused a two-fold increase in the Cu(II) peak area measured at pH 1.5 (but again no change in that measured at pH 4.8). Humic substances had no affect on the Pb(II) stripping peak at pH 1.5 or 4.8. These measurements were performed by use of procedure 1.

The mechanism for the large increase in Cu(II) peak area in the presence of humic substances at pH 1.5 is not known. Increasing the concentration of humic substance (from

0.3 - 3 ppm) had no further effect. This observation could be of some analytical utility. Provided that the adsorption effect of the humic substances is known, their addition to an acidic Cu(II) solution could enhance the sensitivity of ASV measurements for this metal ion at a TMFE. It is possible that the humic substance is strongly adsorbed on the glassy carbon sites not occupied by mercury and then acts as an ion-exchanger to pre-concentrate Cu(II), thus holding it close to the mercury droplets and facilitating enhanced deposition. In contrast to ion exchange, the metal complexation capacity of humic substances will be quite small at pH 1.5, but there may be some humic moieties with a very high affinity for Cu(II) (e.g. ligands containing nitrogen or sulphur donor groups). The higher affinity of humic substances for Cu(II) (Saar & Weber, 1982) may explain why an equivalent effect is not observed for Pb(II). A similar effect is not expected (and was not observed) for tannins because they contain no carboxyl groups. Peak enhancements at a TMFE have been reported in the presence of organic colloids (Ugapo & Pickering, 1985) and a range of surfactants (Beveridge & Pickering, 1984).

Supporting evidence for preconcentration of Cu(II) by humic substances at the mercury surface may be provided by studies on the reduction of Cd(II), Pb(II), and Zn(II) in the presence of long-chain fatty acids at a HMDE (Krznaric et al., 1983). In the presence of fatty acids, the peak height obtained by DP polarography at pH 1.7 was up to ten times greater than that obtained in their absence. It was proposed that this increase is due to the "enhanced adsorption of metal ions at the electrode surface". Although an accumulation of Cd(II) in the adsorbed layer of humic and fulvic acids has not been observed (Cosovic, 1985), an accumulation of Cu(II) should not be discounted since Cu(II) forms much stronger complexes with humic substances than does Cd(II).

Humic substances did not facilitate deposition of Cu(II) or Pb(II) on a bare glassy carbon electrode (Section 7.5.2). In addition, a cathodic scan following adsorption at +0.15 V indicated no evidence for adsorbed Cu(II)-fulvic acid complexes at a TMFE at pH 1.5. (However, a TMFE is quite insensitive for CSV measurements (Batley & Florence, 1976b)). Further, no corresponding increase in Cu(II) stripping peak in the presence of humic substances is observed at a TMFE at pH 4.8 (where humic substances will have a much greater metal complexation capacity). Perhaps this is because the deposition of metals on bare

glassy carbon is much more efficient at pH 4.8 than at pH 1.5 (Section 7.5.1) such that any contribution to deposition from humic substances is less apparent.

The lower susceptibility of a TMFE to adsorption interferences (as compared to a HMDE) has been reported by other workers (Batley & Florence, 1974). The reason for this behaviour is not yet well understood. The geometry of the electrode surface and the diffusion layer, and the surface-to-volume ratio of a TMFE are quite different from those of a HMDE. However, these differences "do not satisfactorily explain why the HMDE and TMFE should behave in a different manner towards insoluble films on the electrode surface" (Batley & Florence, 1974). Smart and Stewart (1985) also reported that surfactants exert different effects on the response of a TMFE as compared to a HMDE. The present work has indicated that with a TMFE the presence of a second electrode surface (the glassy carbon sites not occupied by mercury) may be important in understanding this phenomenon (Section 7.5).

After obtaining a reproducible stripping peak at a TMFE (i.e. after two or three deposition-stripping cycles), successive deposition-stripping cycles on addition of humic substances resulted in reproducible stripping peaks. This indicates that the adsorption of humic substances on a TMFE was rapid and did not increase over time. However, "carry-over" effects were observed. After the TMFE had been exposed to humic substances then rinsed and transferred to a humic-free solution it continued to respond as if humic substances were present. This has also been reported by Hume and Carter (1972).

Tannins and Triton X-100

As was observed at a HMDE, tannins and Triton X-100 had a much greater impact on Cu(II) and Pb(II) stripping peaks at a TMFE than did humic substances (procedure 1). They also had a small effect on stripping peaks even when they were present only in the stripping step (Table 7.8).

At pH 1.5, the effect of tannins and Triton X-100 on the stripping step alone was much less than that when the compounds were present during deposition and stripping (in contrast to what was observed at a HMDE). The Cu(II) peak area at pH 1.5 was decreased significantly on deposition and stripping in the presence of tannins and Triton X-100; this was also observed for tannins at a HMDE. The Pb(II) stripping peak at a TMFE at pH 1.5 was

decreased on deposition and stripping in the presence of these substances (Table 7.7); in contrast, a small increase was observed at a HMDE (Table 7.5).

At pH 4.8, the effect of the condensed tannin on the Cu(II) and Pb(II) stripping peaks at a TMFE was approximately the same as that observed at a HMDE.

As was observed at a HMDE, the Cu(II) and Pb(II) peaks were shifted to more positive potentials in the presence of tannins and Triton X-100.

With Triton X-100, evidence was obtained for slow adsorption on the TMFE and/or changes in the nature of the adsorbed species over time (consistent with observations at a HMDE). Successive deposition-stripping cycles in the presence of Triton X-100 showed a continual decrease in peak area and shift in peak potential. As noted above, this is not due to slow changes in solution chemistry. Further, it is not clear why the effect of Triton X-100 on Cu(II) and Pb(II) at pH 1.5 should decrease as the concentration of Triton is increased (Table 7.7). (It was established that the Triton X-100 sample did not contain any measurable metal impurities).

Nafion-Coated Thin-Mercury Film Electrode (NCTMFE)

Humic Substances

A Nafion coating appears to completely eliminate the adsorption effects of humic substances at a TMFE, with both DC and DP modes (procedure 1). However, the only adsorption effect exhibited by humic substances at a non-coated TMFE was an increase in the Cu(II) stripping peak at pH 1.5. That is, for measurements in the presence of humic substances at pH 4.8 a NCTMFE offers no advantages over a TMFE.

The processes occurring at a NCTMFE are not yet well understood. The Nafion film may serve to reduce the overpotential of the electrode surface (Dr H.K.J. Powell, pers. comm. 1990) and/or it may act as an ion exchanger and hold metal ions close to the mercury droplets and thus facilitate more efficient deposition. However, in the present work it was observed that the Cu(II) DC-ASV peak current measured at a NCTMFE at pH 4.8 was the same as that measured at a non-coated TMFE (this has also been reported by Hoyer et al. (1987). Hence, the "ion-exchange" proposal seems unlikely.

Tannins

At pH 1.5, the Nafion coating appeared to eliminate any tannin adsorption effects. At pH 4.8, the condensed tannin had a small impact on the Cu(II) stripping peak, but no effect on the Pb(II) peak area, when either DC or DP mode was used (procedure 1).

Triton X-100

By use of procedure 1 it was established that Triton X-100 did have a significant impact on the stripping peaks measured at a NCTMFE (Table 7.9). In contrast to what was observed at a TMFE (Table 7.7), the Pb(II) and Cu(II) stripping peaks were *increased* in the presence of Triton X-100 at a NCTMFE. The increase in Cu(II) peak area was similar at pH 1.5 and 4.8; for Pb(II) the stripping peak at pH 1.5 in the presence of Triton X-100 was two times greater than that at pH 4.8.

Since a significant decrease in copper(II) stripping peaks was observed in the presence of Triton X-100 at a TMFE, the results obtained at a NCTMFE may suggest that the Nafion film has prevented this surfactant from coming into contact with the glassy carbon sites and the mercury film. Triton X-100 is unlikely to have a significant complexation capacity for Cu(II) or Pb(II).

Nafion contains large segments of uncharged moieties which allow for extensive hydrophobic interactions (Szentirmay & Martin, 1984). Hence, it is possible that Triton X-100 permeates the Nafion film, perhaps dissolving in the hydrophobic domains of the polymer, and thus enhances the affinity of Nafion for metal ions, possibly by enhancing the diffusion of metal ions through the channels in Nafion and/or changing the solvation of the ion exchange sites on the Nafion polymer. This effect of Triton X-100 on Nafion may be time dependent. For example, when Triton X-100 was present only in the stripping step at pH 1.5, the Pb(II) stripping peak was increased by only 10% of the amount that occurred on deposition and stripping in the presence of Triton X-100. Stripping peaks for subsequent deposition-stripping cycles at a NCTMFE in the presence of Triton X-100 were reproducible.

Nafion seems to have a greater association with Pb(II) than with Cu(II). The observation that the Pb(II) peak area for stripping at a NCTMFE in the presence of Triton X-100 (with no preceeding deposition step) was much greater than that for Cu(II) supports this proposal. Holding the electrode potential at 0 V for several minutes did not reduce this

residual peak. Further, whereas at a bare glassy carbon electrode in the presence of equal concentrations of Cu(II) and Pb(II) the Cu(II) DC-ASV stripping peak was 2.1 times greater than that for Pb(II), at a Nafion-coated glassy carbon electrode (with no mercury film) the Pb(II) stripping peak was 30% greater than that for Cu(II). The affinity of Nafion for Pb(II) has also been reported by Dong and Wang (1988a). Incorporation of a crown ether (DC18C6) into the Nafion film raised the sensitivity of a Nafion/bare glassy carbon electrode for Pb(II) (Dong & Wang, 1988a). Also, enhanced Pb(II) DC-ASV peak currents in the presence of Triton X-100 at a NCTMFE have been observed by Dr G.E. Batley (pers. comm., 1990).

Triton X-100 (2 ppm) caused a small increase (6%) in the square-wave Cd(II) peak current at a NCTMFE (Hoyer et al., 1987).

Interestingly, with DP mode a significant *decrease* in the peak current measured at a NCTMFE was observed in the presence of Triton X-100 at pH 4.8 (Table 7.9). Other authors have reported that Triton X-100 slows down the kinetics of processes occurring at a mercury surface (Jacobsen & Lindseth, 1976; Cosovic, 1985).

7.4.5 Summary of Adsorption Effects

In comparison to tannins and Triton X-100, the effect of humic substances on the electrodes studied appears to be relatively minor. Any adsorption effect exhibited by humic substances affected only the deposition step and resulted in a decrease in stripping peak (except for Cu(II) at a TMFE at pH 1.5) and a negative shift in peak potential. Adsorption effects were less severe at a TMFE than at a HMDE.

In contrast, tannins and Triton X-100 had a significant impact on both the deposition and stripping steps. The peak potential was always shifted to more positive voltages and both decreases and increases in stripping peaks were observed (depending on the pH and the particular substance studied).

The deposition potential used to study the effects of humic substances on Cu(II) and Pb(II) stripping peaks at a HMDE and a TMFE had no effect on the results obtained. Further, humic substances had the same impact on the Cu(II) and Pb(II) stripping peaks whether these metals were measured individually (using a deposition potential of -0.6 V and -0.9 V respectively) or simultaneously (-0.9 V deposition potential).

Although Triton X-100 is often used as a "model" substance in studies on the effects of adsorption of surfactants on electrode surfaces (Mann & Florence, 1987a; Kubiak & Wang, 1989a), the present work indicated that it may grossly overestimate the behaviour of naturally occurring humic substances.

A NCTMFE appeared to protect the electrode from the adsorption effects of relatively large molecules (either anionic e.g. humic substances or nonionic e.g. tannins). However, hydrophobic species such as Triton X-100 had a significant impact on the stripping peaks.

The processes occurring at a NCTMFE are not yet well understood. It is possible that small molecules are able to penetrate the Nafion film and have a deleterious effect on the mercury surface. For example, a Nafion film cannot protect a TMFE from the adsorption effects of whole blood. Although Hoyer and Florence (1987) reported that Pb(II) in whole blood could be determined directly at a NCTMFE, in 0.5 mol L^{-1} HCl medium, it was observed in the present work (and by Dr H.K.J. Powell (unpublished results)) that, in the presence of whole blood, the Pb(II) and Cu(II) stripping peaks decreased substantially on successive deposition-stripping cycles until eventually zero signal was obtained. Similarly, a NCTMFE did not allow partially digested biological samples to be analyzed satisfactorily, i.e. adsorption problems were observed (Dr G.E. Batley, pers. comm., 1990). The distribution of mercury in the Nafion film is also yet to be established unequivocally (Section 7.6.1).

In conclusion, the adsorption of organic substances on a HMDE, a TMFE, and a NCTMFE is complicated and does not follow a simple, predictable pattern. The adsorption characteristics of each compound must be determined individually.

7.4.6 Apparent Lability of Metal-Humic Complexes

The ASV labile fraction of metal ions in natural waters has been correlated with their bioavailability and toxicity (Florence, 1986). Humic substances are important complexors of metal ions in the environment; hence, they may control the concentration of free and labile metal ions in soils and natural waters.

There has been some controversy in the literature as to the optimal pH for the determination of labile metal (Skogerboe et al., 1980). Because the act of measuring a sample by ASV will perturb natural equilibria, Florence and Batley (Skogerboe et al., 1980) argued that it is best to perform lability measurements in a buffered, well-defined system to allow

results from different samples to be readily compared. Although it has been suggested that the addition of acetate ions to a sample may displace other ligands (Skogerboe et al., 1980), Mann and Florence (1987b) found that concentrations of acetate ions up to 0.15 mol L^{-1} had no significant effect on lability measurements.

A similar controversy has prevailed over the use of TMFEs with *in situ* mercury deposition for the determination of labile metal. Although mercuric ions form stable complexes with many ligands and thus may alter the original speciation of a sample (Skogerboe et al., 1980; Kramer et al., 1984), Mann and Florence (1987b) reported that up to 8×10^{-5} mol L^{-1} Hg(II) had no significant impact on the apparent DP-ASV labile fraction of Zn(II), Cd(II), Pb(II), and Cu(II) in fresh waters. However, the presence of Hg(II) has been found to increase the apparent concentration of DC-ASV labile metal by exchange of Hg(II) ions with nonlabile metal complexes (Brihaye et al., 1983; Powell & Florence, 1990). The results reported for DP -ASV measurements (Mann & Florence, 1987b) reflect the sensitivity of this technique to factors other than complex lability (Powell & Florence, 1990). As recommended by Powell and Florence (1990), DC-ASV at a pre-plated TMFE was used for lability measurements in the present work.

Experimental conditions should be such that the ASV-labile fraction is governed only by factors which affect the deposition step (Morrison et al., 1990). The macromolecular humic substance-metal ion system is complex and many factors may contribute to the deposition step (diffusion coefficient of ML (Cleven et al., 1986), lability of ML, adsorption of humic substance (Opperman et al., 1988), excess L generated at the electrode surface (Stolzberg, 1977)) and/or the stripping step (excess M^{2+} generated at the electrode surface (Buffle, 1981), adsorption of humic substance, formation of multiple complex species of varying lability (Gregor & Powell, 1988a)).

In a study of metal-fulvic acid equilibria by DC-ASV at a HMDE, Gregor and Powell (1988a) attempted to isolate the variables which contribute to the reduction and oxidation steps. These authors proposed an equation for the apparent nonlabile fraction (α) of metal-fulvic acid complexes at pH 4.8, *viz:*

$$\alpha = 1 - \frac{\text{pk area at pH 4.8}}{\text{pk area at pH 1.5}}$$

In the present work some inadequacies were found in this equation. Implicit in the Gregor-Powell equation is the assumption that the adsorption effects of fulvic acid will be the same at pH 1.5 and 4.8. The present work indicated that this is true for Cu(II) but not for Pb(II). This equation also requires that the sensitivity of the electrode is equal at pH 1.5 and 4.8; this is not a valid assumption.

In the present work, the Cu(II) DC-ASV peak area measured at a HMDE at pH 4.8 (0.01 mol L^{-1} acetate buffer) was found to be the same in the presence and absence of citric acid, indicating that the Cu(II)-citrate complex is 100% labile. However, acidification of the solution to pH 1.5 resulted in a 53% increase in Cu(II) peak area and, according to the Gregor-Powell equation, Cu(II)-citrate would be 35% nonlabile. This increase in stripping peak on acidification is not due to labilization of polymeric Cu-hydroxy species which may have formed in the stock Cu(II) solution (re-buffering the solution to pH 4.8 resulted in the same initial stripping peak). Schönberger and Pickering (1980) ascribed this phenomenon to the lower conductivity of an acetate buffer solution, the increased dissociation of Cu(II)-acetate complexes, and the displacement by protons of acetate ions adsorbed on the electrode surface (which may impede electron transfer) at low pH. Therefore, when the apparent lability of a metal species is being determined the measurements in the presence and absence of ligand must be performed at the same pH.

Further, it is important that the amount of metal which is deposited during the stripping step (i.e. the quiescent time and the time of the anodic scan) is subtracted from the measurements.

In the present work, an alternative equation for calculating the apparent nonlabile fraction of humic and fulvic acid metal complexes at a HMDE, a TMFE, and a NCTMFE (at any given pH) has been developed, *viz:*

$$\alpha = 1 - \frac{\text{corrected pk area in the presence of HS}}{\text{pk area in the absence of HS}} \qquad (1)$$

An important feature of this equation is that the peak area measured in the presence of humic substance is corrected for the adsorption effects reported above, and the metal deposited during the quiescent stage and the stripping step is subtracted from all measurements.

An example of this calculation is now given for the determination of the lability of Cu(II)-FA4 complexes at a HMDE at pH 4.8 (peak areas are given in arbitrary units):

pk area (4 min deposition) from 1.2×10^{-7} M Cu(II) = 0.0886

pk area for scan-only = 0.0073

pk area corrected for scan-only = 0.0813

pk area (4 min deposition) from Cu(II) + 1 ppm FA4 = 0.0271

pk area for scan-only = 0.0026

pk area corrected for scan-only = 0.0245

From Table 7.2, the DC-ASV peak area at a HMDE is suppressed by 13% in the presence of FA4 at pH 4.8. Therefore, the peak area for deposition from a Cu(II)-FA4 solution corrected for humic substance adsorption = 0.0245 x 1.15 = 0.0282.
Therefore, the percentage of nonlabile Cu(II) complexes formed by FA4 at pH 4.8 is given by:

$$\left(1 - \frac{0.0282}{0.0813}\right) \times \frac{100}{1} = 65\%$$

It is important to note that this equation cannot determine whether the reduced peak area in the presence of humic substances results from the formation of nonlabile complexes or from the presence of complexes with a low diffusion coefficient. However, schemes for the rigorous classification of macromolecular ligand-metal ion systems are extremely complex (van Leeuwen et al., 1989a). Indeed, Esteban et al. (1990) considered theoretical interpretations of voltammetric polyelectrolyte-metal ion titrations (van Leeuwen, 1987) to be unsuitable for describing measurements in the presence of humic substances.

Given that humic substances are ill-defined and heterogeneous with respect to thermodynamics and kinetics of metal binding as well as diffusion coefficients (Esteban et al., 1990), the results presented herein are *not* considered as *absolute* values. Rather, the equation used in the present work is proposed to provide a convenient basis for *comparing* the 'apparent' lability of metal complexes formed by different humic samples in which the factors known to interfere in the measurements are minimized or controlled.

The following sections discuss the lability of copper(II) and lead(II) complexes with humic substances at the hanging mercury drop, the thin-mercury film, and the Nafion-coated thin-mercury film electrodes. These measurements were performed by use of procedure 2.

Lability of Metal-Humic Complexes at a HMDE

The percentage of nonlabile Cu(II) and Pb(II) complexes formed by FA4 and FA2 measured in the present work and calculated according to equation (1) (Table 7.10) is much greater than that reported by Gregor and Powell (1988a). An attempt was made to replicate data under their experimental conditions (i.e. slow rate of solution stirring, and using their equation to calculate the apparent complex lability). However, it was discovered that their data were not measured at pH 4.8 as reported. These authors added $HClO_4$ to the test solutions (before buffering) to effect initial dissolution of metal-hydroxy polymers which were thought to have formed in the stock Cu(II) solution (even though it was stored at pH 4.0). The presence of this strong acid lowered the pH of the acetate buffer solution.

Under Gregor's (1987) reported experimental conditions (0.01 mol L^{-1} acetate buffer, 0.012 mol L^{-1} $HClO_4$; measured pH = 3.8) the percentage of apparent nonlabile Cu(II) complexes formed by FA4 (46%; Gregor & Powell, 1988a) was reproduced in the present work (48%). The value reported for FA2 (29%; Gregor & Powell, 1988a), however, was not reproducible (45%). Gregor & Powell (1988a) did not duplicate their result for FA2. They reasoned that the greater proportion of nonlabile Cu(II) complexes formed by FA4 was due to the higher nitrogen content of this fulvic acid sample.

In the present work no significant difference was observed between the percentage of apparent nonlabile Cu(II) and Pb(II) complexes formed by FA4 (2.3% N), FA2 (0.6% N) and SHHA (4.7% N) at pH 4.8 and 5.5; Table 7.10. There was a decrease (from 39 to 25%) in the lability of Cu(II)-SHHA complexes on raising the pH from 4.8 to 5.5; for Pb(II)-SHHA complexes, the lability decreased from 62 to 48%.

These results may indicate that, globally, the lability of metal complexes formed by humic and fulvic acids are very similar and are not sensitive to small differences in composition of the humic samples.

Effect of Reverse-Phase Chromatographic Fractionation of Fulvic Acid

The components of FA4 which were not adsorbed on a Sep-pak C_{18} cartridge did not adsorb significantly on the mercury drop electrode at pH 1.5 or 4.8 (procedure 1) (Section 7.4.4). Therefore, it was of interest to investigate whether removal of the "hydrophobic" components of fulvic acid had any measurable impact on the lability of the copper(II) complexes formed by the remaining "hydrophilic" moieties.

The "hydrophilic" fulvic acid components formed Cu(II) complexes which were 53% nonlabile at pH 4.8, compared with 67% for the whole FA4 sample (Table 7.10). This greater lability of the "hydrophilic" complexes is interesting. Several possibilities arise: (i) the "hydrophobic" fulvic acid-copper(II) complexes may be less labile and/or have a lower diffusion coefficient than do the "hydrophilic" complexes, and/or in the unfractionated fulvic acid (ii) the "hydrophobic" moieties may block labile Cu(II) complexation sites (Antworth et al., 1989), or (iii) the hydrophilic and hydrophobic components may form ternary complexes.

Molecular Size Fractionation of Humic Substances by Ultrafiltration

The lower molecular size components of fulvic and humic acid formed Cu(II) complexes which had higher apparent lability than those formed by the whole sample or by the higher molecular size fraction (Section 7.3.4). The differences in apparent lability may result from a change in the mean diffusion coefficient of the Cu(II) complexes.

Ternary Complexes

The possibility that humic substances form ternary copper(II) complexes with nitrogen-donor ligands was investigated. The formation of such Cu(II) species may be of environmental significance. In soils and natural waters the presence of a wide variety of non-humic ligands may result in the actual lability of "humic substance"-metal complexes being significantly lower than those formed by isolated humic fractions.

The lability of fulvic acid-Cu(II) complexes in a mixed acetate buffer/NH_3 solution, pH 6.3 (0.01 mol L^{-1} acetate buffer (pH 4.8), 7 x 10^{-3} mol L^{-1} NH_3) was measured and compared with that obtained in the absence of NH_3. The Cu(II) complexes formed by FA4 (2.4 x 10^{-3} mg mL^{-1} FA, 9.5 x 10^{-7} mol L^{-1} Cu(II)) were 95% nonlabile in the presence of NH_3, compared with 80% in acetate buffer at the same pH. (The Cu(II)-ammine complex was 100%

labile). This decrease in apparent lability of Cu(II)-FA4 complexes in the presence of NH_3 may represent the formation of ternary FA-Cu(II)-ammine complexes which are less labile and/or have a lower diffusion coefficient than do FA-Cu(II) species. However, quite a high concentration of NH_3 was used in these experiments.

The presence of aspartic acid, at a more environmentally significant concentration (10^{-6} mol L^{-1}), had no effect on the apparent lability of Cu(II)-SHHA complexes at pH 4.8. However, calculations based on published stability constants indicated that only 0.5% of the total Cu(II) in solution would be complexed by aspartic acid at pH 4.8. It is possible that ternary complex formation by humic substances becomes significant only at higher pH (e.g. in seawater, pH 8.2).

Lability of Metal-Humic Complexes at a TMFE

The apparent lability of humic and fulvic Cu(II) complexes was measured at the laboratory-built TMFE (with solution stirring) at pH 4.8 and 5.5; Table 7.11. The proportion of labile complexes formed by all samples at each pH is similar. On average, the Cu(II) complexes formed by each sample are 90% labile at pH 4.8 and 74% labile at pH 5.5. These values are in contrast to those obtained at a HMDE (Table 7.10).

These observations highlight the operational definition of lability.

Several factors may contribute to the very different results obtained at a TMFE as compared to a HMDE. Firstly, humic substances have different adsorption effects at these electrodes (Section 7.4.4). However, the equation used in the present work to calculate apparent labilities is meant to compensate for this contribution to the stripping peak. Secondly, in addition to the different *geometry* of the diffusion layer (spherical diffusion at a HMDE; linear diffusion at a TMFE), the *thickness* of the diffusion layer will also be different at each electrode. Indeed, Davison (1978) has proposed that the effective diffusion layer thickness is the critical parameter for determining which species in solution will be detected by ASV.

Importantly, in addition to mercury droplets, a significant proportion of the surface area of a TMFE is comprised of glassy carbon sites (Stulikova, 1973) (this is discussed further in Section 7.5). As proposed above, humic substances may facilitate deposition at a TMFE by adsorbing on the glassy carbon sites and holding metal ions close to the mercury by acting as an ion exchanger. If this is so, then the effective diffusion layer may have no significant

impact on the apparent lability of metal-humic complexes. That is, the metal ions do not need to traverse the diffusion layer as they are already at the surface of the electrode. If such a mechanism is in effect then a greater percentage of complexes would be detected as "labile" at a TMFE than at a HMDE; this was observed.

Some of the discrepancy between lability measurements at a HMDE and those at a TMFE could represent "inter-electrode reproducibility". For example, there is a significant difference between the apparent lability of Cu(II)-FA4 complexes measured at the laboratory-built TMFE (Table 7.11) and that measured at the Metrohm rotating TMFE (Table 7.12). The relative efficiency of ASV deposition with solution stirring as compared to rotation of the electrode may account for this difference (Acebal & Rebello, 1983). This observation is also consistent with a smaller effective diffusion layer at the rotating electrode. This fact again highlights the operational nature of these measurements, and restricts interpretations to a comparative level.

The possibility that ionic strength may affect lability was investigated. Measurements at a HMDE were performed at an ionic strength of 0.01 mol L^{-1}, while 0.1 mol L^{-1} was used at a TMFE (to minimize the deposition which occurred during the stripping step). However, the apparent lability of Cu(II)-FA4 complexes measured at a HMDE was the same at an ionic strength of 0.01 mol L^{-1} and 0.1 mol L^{-1}. Therefore, the ionic strength used for these experiments cannot account for the different labilities measured at the HMDE and the TMFE.

Lability as a Function of Diffusion Layer Thickness

Theoretically, the thicker the diffusion layer, the higher the percentage of labile complexes. Thus, the apparent lability of a partially labile complex is expected to be greater at a HMDE than at a TMFE (where the faster rate of electrode rotation effects a thinner apparent diffusion layer). However, in practice the opposite was observed. Further, Cu(II) and Pb(II) complexes with FA4, FA2, and SHHA had the same apparent lability at a HMDE when either "slow" (*ca.* 400 rpm) or "fast" (*ca.* 700 rpm) stirring was used (*ca.* 30-40%). Therefore, factors other than the thickness of the diffusion layer must be important in governing the amount of labile metal which is detected at these electrodes.

Varying the rate of electrode rotation of a TMFE provides a precise and reproducible way of varying the diffusion layer thickness. The apparent lability of Cu(II) and Pb(II) humic

complexes did not change when the electrode rotation speed, and hence the diffusion layer thickness, was altered (Tables 7.12 and 7.13). It is possible that the "ion-exchange" mechanism can account for this observation at a TMFE. However, this cannot be the reason why the lability measured at a HMDE was not affected by the rate of stirring of the solution.

Alternatively, the metal-humic species detected by ASV may be 100% labile but with a significantly smaller diffusion coefficient than for the free (aqua) metal ion; hence the apparent lability would not be affected by changes in the thickness of the diffusion layer. Indeed, Pb(II) complexes with pedogenic aquatic fulvic acids are 100% labile (Buffle, 1988). Inert complexes may also be formed which are not detected by ASV under any experimental conditions.

Levich plots (i_p *vs* $w^{1/2}$) were linear in the presence and absence of humic substances. This is indicative of a diffusion controlled process. That is, association/dissociation kinetics are not rate limiting for the voltammetric signal and the stripping peak is controlled by a mean diffusion coefficient.

For comparison, the DP-ASV lability was also measured. Copper(II)-FA4 complexes were 26% DP-ASV nonlabile at pH 4.8 at the laboratory-built TMFE with solution stirring (c.f. 10% DC-ASV nonlabile). With DP mode, humic substances had no adsorption effect on the Cu(II) peak current at pH 4.8 (procedure 1). Hence, even in the absence of adsorption interferences, the differences between DC-ASV and DP-ASV lability again highlight the sensitivity of the DP waveform to factors other than complex lability.

Effect of Fractionation of Humic Substances

As was described above for the HMDE, the effect of Sep-pak C_{18} fractionation and molecular size fractionation by ultrafiltration on the apparent lability of Cu(II)-humic and fulvic complexes at a TMFE was investigated. No change in lability of the copper(II) complexes was observed following these treatments. It is not known why the apparent lability of the fractionated humic substances should be the same as that measured for the whole sample at a TMFE, whereas at a HMDE significant differences were observed.

Lability of Metal-Humic Complexes at a NCTMFE

Data obtained using the laboratory-built electrode with solution stirring were not reproducible; Table 7.14. When mercury is plated through Nafion, very reproducible experimental parameters may be critical in forming a mercury film with a constant film thickness and with the mercury evenly distributed over the glassy carbon surface. The rotating electrode provides much more reproducible deposition conditions.

Copper(II) complexes with SHHA and FA4 were much less labile at pH 4.8 at the rotating NCTMFE (Table 7.15) than at the rotating TMFE (Table 7.12), whereas at pH 5.5 the labilities were the same (Table 7.16); the reason for this is not known.

Morrison and Florence (1989b) considered the diffusion layer to be contained beneath the Nafion film. The film matrix will be negatively charged; hence, negatively charged complexes, such as Cu(II)-humates, may be repelled from its surface resulting in a lower apparent lability (Morrison & Florence, 1989b). In an earlier paper however, Hoyer et al. (1987) calculated that the Nafion coating would be considerably thinner than the diffusion layer. The present work supports the latter proposal, i.e. the diffusion layer extends beyond the Nafion film. For example, Cu(II)-citrate was 100% DC-ASV labile at a NCTMFE. If the diffusion layer was contained beneath the Nafion film then some reduction in the apparent lability of this negatively charged complex would be expected at a NCTMFE. Further, Morrison and Florence (1989b) reported that Cu(II)-fulvic acid complexes (which will be negatively charged) have similar DP-electroactivity at a TMFE and a NCTMFE, suggesting (contrary to their above proposal) that the diffusion layer does extend beyond the Nafion film thickness.

The lower lability of Cu(II)-humic complexes at a NCTMFE could also arise from a lower effective diffusion coefficient for the Cu(II) species at this electrode. For example, oxygen has a lower diffusion coefficient in the presence of a Nafion coating (Anson et al., 1985). However, diffusion coefficients of ions in Nafion are generally determined after the coated electrode has been equilibrated with the species of interest for several hours. Further, a wide range of diffusion coefficients have been reported for structurally similar cations incorporated in Nafion films. Buttry and Anson (1983) explained this observation in terms of the unusual structural features of Nafion that include both hydrophilic and hydrophobic phases between which incorporated substances may partition. Ions of lower charge diffuse more

slowly in the hydrophobic phase than in the hydrophilic domains (Martin & Dollard, 1983). The hydrophilic zones in Nafion are comprised of ionic sulphonate groups clustered with polar solvent molecules. These clusters are surrounded by a hydrophobic fluorocarbon matrix, with channels connecting the clusters (Faulkner, 1984; Waller, 1989).

In the present work, the NCTMFE was not equilibrated with the Cu(II)-humic solution for an extended period before the lability measurements were made. It is probable that the lower lability of Cu(II)-humic complexes observed at a NCTMFE is due to a lower diffusion coefficient for these complexes through the *channels* in the Nafion (as opposed to through the bulk of the polymer). Indeed, Levich plots (i_p versus $w^{1/2}$) were linear at a NCTMFE in the presence and absence of humic substances at pH 1.5 and 4.8, indicating a diffusion controlled process. The slope of the Levich plot was reduced in the presence of humic substances. In the presence of fulvic acid the slope of the Levich plot for Cu(II) at a TMFE was reduced by 19%; at a NCTMFE the reduction in slope was 43%. This is consistent with a lower effective diffusion coefficient for metal-fulvic species at a NCTMFE and/or exclusion of negatively charged complexes by the Nafion film. Although, in the presence of SHHA the slopes of the Levich plots at a TMFE and a NCTMFE were reduced by a similar amount (*ca.* 20%).

7.4.7 Summary

Humic substances adsorb on a HMDE and suppress the Cu(II) and Pb(II) stripping peaks. In contrast, their adsorption on a TMFE was minimal with the only effect being an increase in the Cu(II) peak area at pH 1.5. Studies on the adsorption characteristics of tannins and Triton X-100 indicated that these substances are not suitable models for humic substances. The effects of organic substances on the electrode response cannot be predicted.

Humic-metal complexes have a much higher apparent lability at a TMFE than a HMDE. Their lability was not dependent on the diffusion layer thickness at any of the electrodes studied.

A Nafion film appeared to prevent the adsorption of organic compounds on a TMFE.

7.5 BARE GLASSY CARBON ELECTRODE STUDIES

As described above, the properties of a TMFE are quite different from those of a HMDE. An attempt was made to gain some further insight into the processes occurring at these electrodes. Specifically, the contribution of the glassy carbon sites to the response of a TMFE was studied. The properties of Nafion films on bare glassy carbon were also investigated.

7.5.1 Deposition and Stripping of Metals

There has been extensive publication on "pretreatment" methods for obtaining active and reproducible glassy carbon electrode surfaces. According to Rice et al. (1990), the fractional density of edge planes, the surface roughness and cleanliness, and the presence of surface functional groups (especially oxides) are the variables which affect the activity of a glassy carbon electrode. Pretreatment procedures may affect any or all of these variables. A variety of pretreatment methods have been reported, including: electrochemical activation (anodization and/or cathodization) (Engstrom & Strasser, 1984; Wang & Tuzhi, 1986; Wang & Lin, 1988; Kepley & Bard, 1988; Mattusch et al. 1989; Bodalbhai & Brajter-Toth, 1990); heat treatment (Kamau, 1988); metallographic polishing (Hoogvliet et al., 1986); carbon arcing (Upadhyay, 1989); and laser activation (Poon & McCreery, 1987). The range of activation techniques reported may reflect the fact that the optimum pretreatment method will be dependent on the electroactive species of interest and the supporting electrolyte used (Engstrom & Strassser, 1984).

In the present work it was of interest to probe the contribution of the glassy carbon surface to the ASV stripping peak measured at a TMFE. Therefore, the pretreatments investigated involved exposing the electrode to the conditions encountered in the TMFE experiments (i.e. holding the potential at negative and positive voltages). It was found that the sensitivity of the glassy carbon electrode at pH 4.8 was enhanced after holding the potential at either a negative (-1.0 V) or a positive (+0.15 V) value; negative potentials resulted in a more reproducible electrode response. Holding the potential at such a value for 10 min resulted in a stable electrode response to deposition and stripping in the presence of metal ions. With a shorter pretreatment time, peak areas increased on successive deposition-stripping cycles.

Wiping the electrode surface with filter paper soaked in ethanol removed this activation. Following pretreatment the response of the laboratory-built electrode was much less reproducible than that of the Metrohm rotating electrode.

A similar enhanced electrode response was obtained whether the pretreatment was performed in the presence or absence of metal ions. Therefore, the activation process must involve some change in the nature of the glassy carbon surface and not formation of an initial metal deposit.

It is interesting to note that the electrochemical pretreatment which gave a sensitive, reproducible response (10 min at -1.0 V) is the same as that inherent in the formation of a TMFE. That is, while the mercury film is being deposited the glassy carbon sites are being activated. It has been reported that the very negative deposition potential used for deposition of the mercury film (-1.0 V) is required to obtain a homogeneous mercury film of closely packed "mercury-microdroplets" of similar size (Stulikova, 1973; Mart et al., 1980). It is possible that the glassy carbon sites need to be activated before mercury deposition can occur. It is probable that the sites on glassy carbon for the deposition of copper are the same as those for the deposition of mercury (Dr G.E. Batley, pers. comm., 1990).

pH Dependence

The response of the bare glassy carbon electrode to the deposition and stripping of metal ions was very pH dependent. At pH 1.5 no significant deposition of Cd(II), Pb(II) or Cu(II) could be effected. Even if the electrode was electrochemically activated at pH 4.8, subsequent acidification resulted in a loss of sensitivity.

In contrast, at pH 4.8 the sensitivity of deposition and stripping of Cu(II) on the Metrohm rotating glassy carbon electrode was the same as that obtained at a TMFE. With the laboratory-built electrode the glassy carbon sensitivity for Cu(II) was *ca.* 60% that of a TMFE. The DC-ASV sensitivity for Pb(II) on the Metrohm glassy carbon electrode at pH 4.8 was *ca.* 30% of that obtained at a TMFE; Cd(II) could not be deposited and stripped on either glassy carbon electrode. That is, the ease of deposition and stripping of metal ions on glassy carbon at pH 4.8 was Cu(II) >Pb(II) >Cd(II). According to Florence (pers. comm., 1990), a deposit of metal on glassy carbon has a lower chemical activity than metal which is dissolved in an amalgam; this results in broader stripping peaks with reduced sensitivity.

In each case all the metal deposited was removed in the subsequent stripping step. Further, there was a linear relationship between the ASV stripping peak measured at pH 4.8 and the concentration of Cu(II) (1×10^{-7} to 4×10^{-7} mol L^{-1}). These results suggest that, especially for Cu(II), deposition and stripping from the glassy carbon sites may make a significant contribution to the ASV stripping peak measured at a TMFE. Indeed, data presented by Stulikova (1973) suggest that only 6% of the surface area of a TMFE is occupied by mercury droplets. According to Florence (pers. comm., 1990), the very low solubility of Cu(II) in mercury means that most of the Cu(II) which is stripped at a TMFE is copper metal, i.e. a solid Cu(II) phase dispersed in mercury rather than a homogeneous amalgam.

This high contribution to the ASV stripping peak from the glassy carbon sites may allow ASV to be performed in the absence of Hg(II). This would simplify the analysis procedure and eliminate any possible interference from Hg(II) exchanging with inert metal complexes (Brihaye et al., 1983; Powell & Florence, 1990). Therefore, the adsorption characteristics of humic substances and the apparent lability of metal-humic complexes at a glassy carbon electrode were investigated.

7.5.2 Adsorption of Organic Substances

At pH 1.5, where the ASV sensitivity of the glassy carbon electrode is very low, the presence of humic substances had no effect on the Cu(II) and Pb(II) stripping peaks. The adsorption of humic substances on glassy carbon and their complexation with metal ions was proposed to explain the enhanced deposition of Cu(II) into mercury at a TMFE at pH 1.5. However, because of the low ASV sensitivity of the glassy carbon sites at pH 1.5 it is possible that humic substances are unable to facilitate deposition. At pH 4.8, humic substances had no effect on the Cu(II) stripping peak when either DC or DP mode was used.

Importantly, these observations imply that the effects of humic substances on the individual glassy carbon and mercury surfaces cannot be combined to predict their impact on a TMFE. For example, at pH 1.5 humic substances had no effect on the Cu(II) stripping peak at a glassy carbon electrode, decreased the stripping peak at a HMDE, yet increased the peak area at a TMFE!

The adsorption characteristics of the condensed tannin and Triton X-100 (0.004%) on glassy carbon were also investigated. At pH 1.5 the tannin had no effect on the Cu(II) and

Pb(II) stripping peaks. However, at pH 4.8 the tannin caused a 75% decrease in the Pb(II) DC-ASV peak area and a 64% decrease in the DP-ASV peak current; the Cu(II) stripping peak was not affected.

Triton X-100 caused a 30% decrease in the Pb(II) DC-ASV peak area and a 67% decrease in the Cu(II) DC-ASV peak area at pH 1.5. At pH 4.8 the Cu(II) DC-ASV stripping peak was decreased by 25% in the presence of Triton X-100; the Cu(II) DP-ASV peak current was completely suppressed.

These observations again demonstrate the non-additive nature of processes occurring at individual glassy carbon and mercury surfaces.

7.5.3 Apparent Lability of Metal-Humic Complexes

Since the sensitivity of the glassy carbon electrode for Cu(II) at pH 4.8 was the same as that of a TMFE, and humic substances had no measurable adsorption effect on glassy carbon, it was of interest to measure the apparent lability of metal-humic complexes at a glassy carbon electrode.

The copper(II) complexes formed by FA4 were 18% nonlabile at a glassy carbon electrode at pH 4.8 (the electrode rotation speed was 2000 rpm), compared with 28% at a TMFE (with the mercury film being deposited for 10 min); Table 7.12. The effect of the thickness of the mercury film on the apparent copper(II)-fulvic acid lability was also investigated. Using a mercury film which had been deposited for 2 min at -1.0 V, Cu(II)-FA4 complexes were 23% nonlabile at pH 4.8; with a mercury film deposited for 20 min the complexes were 27% nonlabile. Hence, the apparent lability of Cu(II)-humic complexes appears to be comparatively insensitive to the amount of deposited mercury (if any) present on the glassy carbon electrode surface.

7.6 NAFION-COATED BARE GLASSY CARBON ELECTRODE (NCGCE) STUDIES

A Nafion-coated glassy carbon electrode (with no mercury film present) had greater sensitivity for the determination of Pb(II) (Dong & Wang, 1988a), silver (Dong & Wang, 1988b) and $Ru(NH_3)_6^{3+}$ (Whiteley & Martin, 1987) than did a bare glassy carbon electrode. The sensitivity of a NCGCE towards deposition and stripping of metal ions and its potential to minimize adsorption interferences from organic compounds were studied in the present work.

During this study it was observed that the response of the laboratory-built NCGCE which was mounted in perspex, decreased continually over time. The Nafion film was apparently peeling from the electrode surface in acid solution. A NCGCE prepared with another laboratory-built electrode (with Tokai glassy carbon) and the Metrohm rotating electrode, which were both mounted in Teflon, gave a stable response in acid solution. This indicates that adhesion of Nafion to a glassy carbon electrode is facilitated by adhesion of the Nafion to Teflon.

With the Metrohm rotating electrode the NCGCE had approximately the same ASV sensitivity for Cu(II) at pH 1.5 as did a TMFE. (In contrast, virtually no Cu(II) or Pb(II) could be deposited on and stripped from glassy carbon at this pH. The response of the NCGCE at pH 1.5 was *ca.* 500 times greater than that at a bare glassy carbon surface). The presence of humic substances or the condensed tannin had no effect on the Cu(II) or Pb(II) stripping peaks measured at a NCGCE at pH 1.5. At pH 4.8 the sensitivity of a NCGCE was similar to that of a NCTMFE.

The question which arises is, how does a Nafion film effect such a large increase in the ASV stripping peak measured at a glassy carbon electrode at pH 1.5? Possible explanations include: (i) the ethanol solvent from which the Nafion film is formed has some activating effect on the glassy carbon surface; (ii) the Nafion film acts as an ion-exchanger and preconcentrates metal ions, holding them close to the electrode surface and thus facilitating deposition; and, (iii) the Nafion film reduces the overpotential of the electrode surface. Holding the NCGCE electrode at -1.0 V for 10 min (which activated a glassy carbon surface at pH 4.8) effected no further increase in sensitivity.

Evaporation of 1 μL of ethanol on the glassy carbon surface had no effect on its ASV sensitivity at pH 1.5. Therefore, the activation by Nafion is not due to some "solvent-modification" of the glassy carbon sites.

If Nafion is acting as an ion exchanger then a similar effect may have been expected at pH 4.8; this was not observed. Hoyer et al. (1987) reported that at 2 x 10^{-7} mol L^{-1} Cd(II), Pb(II) and Cu(II), cation-exchange preconcentration by a Nafion film is insignificant in comparison to diffusion of metal ions through the film during the ASV deposition step (at a NCTMFE). At much higher metal ion concentrations (10^{-5} mol L^{-1}), evidence for preconcentration by Nafion films has been reported; however, ion-exchange was found to be significant only in solutions of ionic strength below 0.1 mol L^{-1} (Guy & Namaratne, 1987). Hence, an ion-exchange process is unlikely to be occurring under the experimental conditions used in the present work (ionic strength of 0.1 mol L^{-1} (KNO_3) and [M(II)] *ca.* 5 x 10^{-7} mol L^{-1}). Therefore, the Nafion film may be facilitating the deposition and stripping of metal ions on glassy carbon by overcoming the overpotential of the glassy carbon surface at pH 1.5.

Much of the variation in the response of glassy carbon and TMFEs may be critically dependent on the "conditioning" of the electrode surface (Dr G.E. Batley, pers. comm., 1990).

7.6.1 Mechanism of Action of Nafion Films

The results of the present work indicated that, in general, a Nafion coating provides no increase in DC-ASV sensitivity over that which is obtained at a bare glassy carbon electrode or a TMFE. The only exception is the huge increase in the ASV sensitivity of a NCGCE over glassy carbon at pH 1.5. (With DP-ASV, however, Hoyer et al. (1987) reported that peak currents were approximately a factor of 2 greater at a NCTMFE than at a TMFE.)

The adsorption of humic substances on a TMFE is minimal with the only effect being an enhancement of the Cu(II) stripping peak at pH 1.5. This does not occur at a NCTMFE which suggests that a Nafion coating prevents organic compounds from coming into contact with the glassy carbon sites not covered by mercury (this was also observed for Triton X-100, Section 7.4.4). However, it has been observed in the present work and by Powell (unpublished results) that Nafion cannot protect a TMFE from the adsorption effects of

components of a whole blood sample. This raises questions about the distribution of mercury within a Nafion film.

The distribution of mercury over the glassy carbon surface and through the Nafion film at a NCTMFE has not been established unequivocally. According to Hoyer et al. (1987), reduction of mercuric ions and growth of the mercury phase will occur at the glassy carbon/Nafion interface. These authors considered the mercury droplets to be attached directly to the glassy carbon surface. Recently, Ugo et al. (1990) have reported data which support this hypothesis; they considered the entire mercury deposit to be contained within the Nafion coating. Nafion has a vertical pore structure consisting of an array of channels. Some of these will be located over bare glassy carbon sites (some of which may be active sites for metal deposition) and others will contain mercury.

Large organic molecules, such as humic substances, may be size excluded by the Nafion film and hence prevented from coming into contact with the mercury and glassy carbon sites. In contrast, smaller molecules, such as low molecular weight proteins in a blood matrix, may be able to enter the pores of the Nafion film and adsorb on both the glassy carbon and the mercury surfaces, thus having a deleterious effect on ASV stripping peaks.

If the mercury film on a NCTMFE has a structure similar to that proposed above then the possibility arises that the Nafion pores could be "filled-up" by use of a long mercury deposition time. This hypothesis was tested using the condensed tannin as a probe. This tannin adsorbed on a TMFE at pH 1.5 causing a decrease in Cu(II) and Pb(II) DC-ASV peak areas (Table 7.7); however, no effect occurred at a NCTMFE. Mercury was deposited for 30 min at -1.0 V through a Nafion film then the electrode was transferred to a solution containing 1.1×10^{-7} mol L^{-1} Cu(II) and Pb(II) at pH 1.5 (0.1 mol L^{-1} KNO_3). Addition of 5×10^{-3} mg mL^{-1} tannin had no effect on the ASV stripping peaks. Therefore, no evidence was obtained for the filling-up of Nafion pores by mercury. Theoretically, there is no limit to the amount of mercury which can be deposited on glassy carbon (Dr G.E. Batley, pers. comm., 1990).

Recently, Ugo et al. (1990) measured the increase in area of mercury at a NCTMFE as a function of the amount of mercury deposited through the Nafion film. They observed that after the deposition of about 20 mC of mercury the deposited mercury layer became so thick that the Nafion film usually broke.

7.7 SUMMARY

The adsorption characteristics of humic substances and the apparent lability of their complexes with Cu(II) and Pb(II) have been studied at a HMDE, a TMFE, a NCTMFE, and a bare glassy carbon electrode. Adsorption effects were less severe at a TMFE than at a HMDE; a Nafion film appeared to eliminate the effects of humic substance adsorption on a TMFE. Tannins and Triton X-100 had a greater impact on the electrode response than did humic substances.

The apparent lability of metal-humic complexes was different at each electrode, which highlights the operational nature of this parameter. Evidence was provided for the apparent low lability of metal-humic species arising from a lower diffusion coefficient than that for the free metal ion.

ASV may be feasible at a glassy carbon electrode in the absence of mercury. Good ASV sensitivity was obtained with a Nafion-coated glassy carbon electrode at pH 1.5 and a bare glassy carbon electrode (or a NCGCE) at pH 4.8. Humic substances did not adsorb on these electrode surfaces to any measurable extent.

To obtain adequate reproducibility of data, a rotating electrode system is recommended.

CHAPTER 8

TOXICITY OF LIPID-SOLUBLE COPPER COMPLEXES TO *NITZSCHIA CLOSTERIUM*: AMELIORATION BY HUMIC SUBSTANCES

8.1 INTRODUCTION

Hydrophobic compounds are persistent in the environment and many are extremely toxic to aquatic organisms. Bioaccumulation of lipid-soluble (hydrophobic) compounds is directly related to their octanol-water partition coefficient (K_{ow}). Where K_{ow} is the equilibrium concentration of the compound in octanol divided by the concentration in water. In general, the less water soluble a compound, the greater its K_{ow} value.

However, the more hydrophobic a compound, the greater its association with humic substances also. Humic-bound hydrophobic pollutants are largely unavailable for uptake by biota (McCarthy, 1983; McCarthy & Jimenez, 1985b; McCarthy, 1989; Servos & Muir, 1989). Hence, humic substances may be very important in moderating the toxicity of hydrophobic pollutants in soils and natural waters. It has been reported that humic substances alter the rates of chemical degradation (Perdue & Wolfe, 1982), photolysis (Zepp et al., 1985; Oris et al., 1990), volatilization (Hassett & Milicic, 1985), transfer to sediments (Caron et al., 1985) and biological uptake of nonpolar organic compounds (McCarthy, 1989).

8.1.1 Transport of Hydrophobic Compounds in the Environment

Humic substances can solubilize compounds that are practically water insoluble, and thereby act as an agent for the mobilization and transport of these substances in soils and natural waters. The presence of humic matter may be expected to *decrease* the amount of any hydrophobic contaminant that will bind to suspended particles or be sequestered in sediments, yet *increase* the amount which remains stabilized within the water column (McCarthy & Jimenez, 1985b). In addition, pollutants may be transported much further downstream from their source,

as loss from the water column by settling out of particles will have less effect in limiting their movement.

Sorption of some nonionic solutes by humic substances is thought to be completely reversible (Chiou et al., 1986). Hence gradual desorption of the organic solute from humus may provide a continuous source of pollutant to the water column, thus posing a delayed biological hazard (Bollag & Loll, 1983). This could lead to organisms being continuously exposed to low levels of pollutants that would otherwise have only limited spatial and temporal distribution. In support of this, Landrum (1989) observed that uptake of polycyclic aromatic hydrocarbons (PAHs) by an amphipod occurred largely *via* sediment interstitial water and was kinetically controlled by desorption from sediment particles and dissolved organic matter.

Under some conditions macromolecules will, on average, move faster through soils than does water; this is due to exclusion of the large molecules from the smaller pores (Enfield & Bengtsson, 1988). Macromolecules should increase the relative mobility of slightly mobile compounds through soils more than that of highly mobile compounds. Consequently, very hydrophobic compounds are expected to have greater mobility under environmental conditions than that predicted ignoring the presence of dissolved organic carbon.

For example, Bengtsson et al. (1987) observed that 500 mg L^{-1} dextran enhances the mobility of hexachlorobenzene by approximately 25%; molecules with greater hydrophobicity, such as humic substances, were predicted to have an even greater impact. This may explain the presence of very hydrophobic pollutants in deep ground water aquifers. However, in contrast, benzo(a)pyrene (BaP) was preferentially associated with humic components which were strongly sorbed to subsoils, especially in iron-rich horizons (McCarthy et al., 1989). Hence the association of BaP with humic substances may not enhance the subsurface transport of this hydrophobic compound.

An aspect which has largely been overlooked in the literature is that colloids (particles with diameters <1 μm) may have a potentially critical role in facilitating contaminant transport in soils and natural waters (McCarthy & Zachara, 1989; Backhus & Gschwend, 1990). Indeed, it has been proposed that macromolecules, or water-immiscible substances such as micelles and colloids,

may enhance the movement of hydrophobic compounds through soils by sorbing substances normally sorbed by stationary soil particles (Enfield, 1985).

Further, the adsorption of humic matter on colloids, such as oxides, layer silicates and calcium carbonate, can impart a negative surface charge, thereby increasing the stability and mobility of these particles. It is recognised that further knowledge of dissolved organic carbon sources and mechanisms in complex environmental systems is required in order to better predict the fate of colloid-mediated contaminant transport through the subsurface (Jardine et al., 1989). A recent study established that sorption of humic substances on kaolinite and hematite clay particles could greatly enhance the sorption of hydrophobic organic compounds by these mineral surfaces (Murphy et al., 1990).

8.1.2 Toxicity of Hydrophobic Compounds to Biota

The primary barrier between an organism and its environment is the cell lipoprotein membrane. Consequently, lipid-soluble (hydrophobic) compounds are extremely toxic to biota. The species which cells seek to avoid most are heavy metal ions, coordination complexes of metal ions, and organometallic complexes (e.g. methylmercury (Boudou et al., 1983)) (Mehlhorn, 1986). Indeed, algae produce a range of exudates designed to chelate heavy metal ions and render them innocuous to the cell (McKnight & Morel, 1979; Xue et al., 1988). There is evidence that 5×10^{-11} mol L^{-1} is the threshold concentration of copper ions that algal species try to maintain by excreting chelating agents (van den Berg et al., 1979). The surfaces of algal cells have a high affinity for copper(II) ions, even in the presence of 10^{-3} mol L^{-1} Ca(II). However, biota appear to have no defence mechanisms to protect themselves against highly toxic lipid-soluble compounds.

Hydrophobic compounds tend to be retained in the adipose tissues of organisms; hence they are often bioaccumulated (Nelson & Donkin, 1985). Thus, nonpolar pesticides, such as DDT, are concentrated in the food chain and may pose an environmental hazard. In contrast, water soluble substances (which may also be toxic) are not bioconcentrated (Thurman, 1985). Nonpolar compounds are concentrated in the hydrophobic centre of cell membranes and may disrupt the ordered structure of the lipids, possibly altering vital lipid-protein interactions.

The hydrophobicity of a compound is often quantified by its K_{ow} value (Chiou et al., 1982; Chiou, 1985). These partition coefficients have been correlated with bioconcentration and with sorption of organic compounds by humic substances in water. Indeed, data presented in an extensive review by Connell (1988) indicates that the octanol-water system is *generally* a good estimator of the organism-water system. However, octanol may not be a good model for the humic-water system. Humic substances can adsorb a significant amount of water, whereas octanol can hold only 5% by weight. Therefore, humic moieties are more polar than octanol and hence comprise a thermodynamically less favourable partitioning phase for hydrophobic compounds (Chiou et al., 1983). The dielectric constant for the hydrophobic part of a biomembrane is approximately 2.0 (Mehlhorn, 1986); whereas that of octanol is 10.3 and n-hexane is 2.0 (CRC Handbook of Chemistry and Physics).

Bioaccumulation of some nonionic species from aqueous solution has been directly related to their K_{ow} value (McCarthy & Jimenez, 1985a; Connell, 1988). This suggests that the mechanism for uptake of lipid-soluble substrates is direct diffusion through the lipoprotein membrane of an organism. That is, bioconcentration of hydrophobic compounds is considered to be a partitioning process between the organisms' adipose tissues and water. Therefore, only thermodynamic parameters which govern the affinities of the compound for the respective phases should affect the bioaccumulation factor (Gobas et al., 1986).

The bioconcentration factor (BCF) increases with decreasing aqueous solubility and/or increasing K_{ow} of a compound, in accord with a partition mechanism. In addition, when BCF values are normalized to the lipid content of aquatic organisms a log-log plot of the BCF and the solute's water solubility (or K_{ow} value) is linear and independent of the particular species studied (Smith et al., 1988).

It is well established that hydrophobic substances are extremely toxic to biota (McCarthy, 1989; Blum & Speece, 1990). Recently, a parallel toxicity for hydrophobic metal complexes has been reported (Ahsanullah & Florence, 1984). This fact has again highlighted the importance of speciation measurements (as opposed to determination of total metal concentrations) in establishing the potential toxicity of metals in the environment. The labile-metal concentration, as determined by anodic stripping voltammetry (ASV) (in differential-pulse mode at a hanging mercury drop

electrode) has been proposed to correlate with the toxic, or bioavailable metal fraction (Florence, 1982; Florence, 1983). However, this relationship between lability and toxicity does not hold for lipid-soluble metal complexes. For a range of lipid-soluble copper complexes, no consistent correlation was observed between the toxic copper fraction measured by algal assay and the labile copper determined by several physico-chemical techniques (Florence et al., 1983).

Hence, other techniques must be developed to measure the toxic lipid-soluble metal fraction. Solvent extraction of aqueous samples with solvents such as chloroform, n-octanol, or hexane-butanol (which simulate the solvent properties of biomembranes) has been used for this purpose (Florence, 1983). More recently, the copper fraction retained on an aluminium hydroxide-coated sulphonic acid cation-exchange resin has been reported to correlate with the toxic fraction as determined by algal assay; lipid-soluble copper complexes were quantitatively adsorbed (Zhang & Florence, 1987). Semipermeable polyethylene tubing containing thin films of lipid showed potential for monitoring the bioavailability of lipophilic contaminants *in situ* (Huckins et al., 1990).

Blust et al. (1986) observed a high degree of correlation between the lipid solubility of copper complexes and their bioavailability to brine shrimps. They proposed that aquatic organisms take up nonionic copper complexes by passive diffusion of membrane-permeable metal species. The fact that dead and live microorganisms exhibit similar bioaccumulation lends further support to this hypothesis (Baughman & Paris, 1981).

Three major transport mechanisms exist for the movement of ionic and molecular species across biological membranes, *viz:* passive diffusion, carrier-mediated pathways, and active transport (Morrison, 1989). Hydrophilic metal ions are transported across lipo-protein membranes *via* facilitated (or "host-mediated") transport in which a receptor molecule in the outer membrane surface binds with a metal ion then diffuses to the interior of the membrane, releasing the metal ion into the cytosol. In contrast, lipid-soluble metal complexes are transported into the cell by the much more rapid process of direct (passive) diffusion. For example, it has been reported that the neutral $HgCl_2$ species permeates lipid bilayer membranes 10^7 times faster than the free metal ion; for $CdCl_2$ the factor was 10^3 (Nelson & Donkin, 1985). Importantly, the ligand also enters the cell. The ligand may be more toxic than the metal ion and/or the metal and ligand

may exert synergic toxicity (Florence & Batley, 1988). The hypothesis that bioaccumulation of hydrophobic compounds occurs primarily by partition into the lipid reservoirs of aquatic organisms is supported by the observation that bioconcentration increases with increased lipid content of the organism. According to Smith et al. (1988), lipid-soluble complexes are usually quite stable and are therefore unlikely to dissociate and provide free metal ion toxicity.

Toxicity of Hydrophobic Compounds: Amelioration by Humic Substances

Much research has been reported on the biotoxicity of metal ions and of hydrophobic pollutants. Contradictory evidence exists for the ameliorating effect of humic substances on the toxicity of hydrophilic metal ions (Winner, 1984; Stackhouse & Benson, 1989). In contrast, hydrophobic organic substrates associated with humic substances are largely unavailable for uptake by aquatic organisms (Landrum et al., 1984; McCarthy, 1989). However, to date the impact of humic substances on the toxicity of hydrophobic metal complexes has not been studied.

Bengtsson et al. (1987) found that a macromolecule at low concentrations (10 mg L^{-1}) will have a significant impact only on very hydrophobic compounds (log K_{ow} >7); however, at higher concentrations of a macromolecule (1 000 - 10 000 mg L^{-1}) an effect will be evident for compounds with log K_{ow} >3. The affinity of an organic solute for humic matter is inversely related to the water solubility and directly related to the K_{ow} of the solute (McCarthy, 1989). Consequently, high molecular weight pollutants, such as PAHs with three or more rings, or polychlorinated biphenyls (PCBs), have a high affinity for humic substances. In the absence of humic substances they are very toxic to biota.

According to Connell (1988), the general characteristics of compounds which are most likely to be bioconcentrated are:

(i) molecular weight <300 Daltons (permeability of biological membranes decreases with increasing molecular size of the molecule).

(ii) log K_{ow} value between 2 and 6 (at very high log K_{ow} values low water solubility of the compound limits transport across the biomembrane (Thomann, 1989)).

(iii) water solubility = 18 to 0.002 mol m^{-3}.

(iv) a very low degree of ionization.

Steric factors may also be important; a loss of membrane permeation has been suggested for hydrophobic molecules with diameters greater than 9.5 Å (Opperhuizen et al., 1985).

The ameliorating effect of humic substances on the toxicity of lipid soluble ligands has been attributed to their influence on the transport of nonionic substrates across the cell membrane. It has been proposed that the polar nature of the polyelectrolytic humic macromolecules overwhelms the lipophilic properties of the associated hydrophobic substrate and thus prevents it from being transported across the biomembrane *via* direct diffusion. Dissolved organic matter does not reduce bioaccumulation by changing the rate of metabolism (McCarthy, 1989).

There has been extensive publication on the reduction in toxicity of hydrophobic pollutants by humic substances. Much of this research has focussed on PAHs and PCBs because these compounds fulfill the requirements for very toxic species (*vide supra*) and are also common pollutants in the environment (e.g. from coal conversion effluents; Herbes et al., 1976). The observation that very hydrophobic compounds, log K_{ow} >6, show reduced BCF values relative to their K_{ow} (Davies & Dobbs, 1984) may be due to strong association of these substrates with humic substances.

McCarthy and Jimenez (1985) studied the uptake of PAHs by bluegills in the presence of dissolved humic materials (10 mg L^{-1}). The rate of uptake of PAH associated with humus was 0 to 10% of that for free dissolved PAH. In another study, Leversee et al. (1983) observed that the bioaccumulation of a series of PAHs by *Daphnia magna* was reduced in the presence of humic matter (0.1 to 1 mg L^{-1}) with the greatest effect exhibited with the most hydrophobic compounds. However, a simple linear relationship between humus concentration and bioaccumulation was not observed (Leversee et al., 1983). A logarithmic relationship between humic concentration and decreased bioavailability of BaP was reported by Kukkonen et al. (1989). In the presence of humic acid (20 mg L^{-1}) a 97% reduction in the bioaccumulation of BaP by *Daphnia magna* was observed (McCarthy, 1983).

Although some general conclusions can be made, it is important to note that the results of bioassays are dependent on many interrelated factors such as the source of humic material, pH, and the particular organism and hydrophobic compound studied. For example, Kukkonen (1989) found that dissolved humus lowered the accumulation of pentachlorophenol by *Heptagenia*

fuscogrisen between pH 4.5 and 7.5 and had no effect at pH 3.5 and 8.5. With *Daphnia magna,* humus lowered the bioaccumulation of dehydroabietic acid (DHAA) between pH 5.5 and 6.5 but had no effect at pH >7. In contrast, humic matter had no effect on uptake of DHAA by *Heptagenia fuscogrisen* at any pH.

Furthermore, the majority of studies on the impact of humic substances on the toxicity of nonpolar compounds to biota have used commercial humic acid samples. As noted in Chapter 1, the use of these materials may grossly overestimate these effects of naturally occurring humic substances. Hence, such results should be viewed with caution.

In summary, the binding of hydrophobic compounds to humic substances reduces their bioavailability. Lipid-soluble substrates have the greatest potential for bioaccumulation and transfer to humans *via* food chains (McCarthy & Jimenez, 1985a,b; McCarthy et al., 1985). The aim of the present study was to investigate if the same effect occurs with hydrophobic metal complexes.

8.1.3 Techniques Used to Probe Humic Substance - Hydrophobic Compound Interactions

A large number of techniques have been used to measure the association of nonpolar organic compounds with humic substances. No one method is suitable for all hydrophobic compounds and each technique has its advantages and drawbacks (Caron & Suffet, 1989). The techniques which have been employed include: gel permeation chromatography, ultrafiltration, reverse phase liquid chromatography, equilibrium dialysis, water solubility enhancement, solvent extraction, gas-phase partitioning (Callaway et al., 1984) and fluorescence.

Gel permeation chromatography (Hayes, 1970; Hassett & Anderson, 1979) and reverse-phase chromatographic techniques (Landrum et al., 1984; Alberts et al., 1989) assume that the humic-bound fraction of a hydrophobic pollutant is completely excluded by the adsorbent column, and that the free pollutant is 100% retained. These techniques are complicated by the fact that humic substances are themselves adsorbed on Sephadex gels (Swift & Posner, 1971; Hine & Bursill, 1984) and on reverse phase media, as in Sep-pak C_{18} columns (Chapter 7). Further, the

binding constants measured with these two techniques vary with flow rate; this is highly suggestive of artifacts and slow re-equilibration (Gauthier et al., 1986).

Dialysis (Carter & Suffet, 1982; McCarthy & Jimenez, 1985a) and ultrafiltration methods (Means & Wijayaratne, 1982) physically separate the free and humic-bound nonpolar compounds *via* semipermeable membranes. Ideally, the hydrophobic (humic) substrate should not interact with the ultrafiltration membrane, and with dialysis the nonpolar species should readily pass through the membrane (otherwise equilibrium may not be reached). It is reported that dialysis membranes strongly sorb some nonpolar compounds (Landrum et al., 1984). However, sorption onto glassware and onto the dialysis membrane should not affect the results of equilibrium dialysis experiments since, at equilibrium, the free and humic-bound compounds are in equilibrium with each other and with the compound bound to the glass or dialysis membrane (Carter & Suffet, 1982; McCarthy & Jimenez, 1985a).

Water solubility enhancement methods (Chiou et al., 1986) measure the effect of humic substances on the apparent aqueous solubility of nonpolar compounds. An increase in solubility in the presence of humic materials is attributed to association of the hydrophobic compound with the dissolved organic matter. Although this technique is applicable to a large number of nonpolar substrates, a disadvantage is that the binding constants are measured at saturation which is usually at a much higher concentration than occurs environmentally.

Solvent extraction involves equilibrating the nonpolar compound with humic substances, followed by extraction with a nonaqueous solvent to recover the unbound nonpolar species (Hassett & Anderson, 1979; Gjessing & Berglind, 1981). It is assumed that the humic-bound species will remain in the aqueous phase and that "free" nonpolar compound will be 100% extracted into the organic phase. It is also assumed that the humic-bound material will not dissociate under the concentration gradient, i.e. any association is considered to be irreversible under the experimental conditions.

Fluorescence appears to be a promising method for measuring the binding of nonpolar compounds to humic substances (Senesi, 1990). Fluorescence polarization has been used to study the interaction of perylene with fulvic acid (Roemelt & Seitz, 1982); fluorescence quenching was used to probe associations between humic substances and PAHs (Gauthier et al., 1986).

Fluorescence has two major advantages over the methods described above. The sensitivity of the technique allows measurements to be made at low concentration; secondly, it reduces the probability of errors because free and bound nonpolar compounds can be distinguished without separation. The use of separation techniques (such as gel chromatography) to determine bound pollutants may disrupt equilibria and lead to incorrect estimates of binding constants (Gauthier et al., 1986). Fluorescence polarization is only applicable in systems where the fluorescence is not quenched by humic materials.

Some of the above techniques have also been used in the present study, but to probe the possible sequestering of hydrophobic metal complexes by humic substances. However, disadvantages were identified with each of these 'indirect' methods (Town & Powell, 1989).

8.1.4 Toxicity of Metal Species to Algae

The most direct way to ascertain whether or not humic substances can ameliorate the toxicity of hydrophobic metal complexes to aquatic organisms is *via* algal assays. Indeed, the very high toxicity of lipid-soluble copper complexes to marine organisms was established by this technique (Florence & Stauber, 1986). Florence and coworkers (CSIRO, Lucas Heights, Sydney, Australia) have published extensively on the mechanisms of toxicity of ionic copper and of copper complexes (with both water- and lipid-soluble ligands) to algae.

Free metal ions are toxic to biota. Water-soluble ligands may reduce copper toxicity. In contrast, lipid-soluble ligands greatly increase the bioaccumulation and toxicity of copper to aquatic organisms. This fact is environmentally important, and it has been proposed that the presence of such ligands should be considered when establishing water-quality criteria (Ahsanullah & Florence, 1984). A British environmental quality standard of 5 $\mu g\ L^{-1}$, expressed as an annual average concentration, has been set for dissolved Cu(II) in coastal and estuarine waters (Apte et al., 1990). Importantly, this legislation recognizes the possible ameliorating effect of water-soluble ligands on Cu(II) toxicity; higher levels of Cu(II) are permissible where complexation by organic ligands is known to reduce Cu(II) toxicity.

The toxicity of a wide range of lipid-soluble copper complexes has been studied; selected values are given in Table 8.1.

Table 8.1: Toxicity of Lipid-Soluble Copper Complexes to *Nitzschia closterium*

ligand[a]	[ligand][b]	[Cu][b]	toxicity index[c]	%solvent extractable Cu[d]
oxine	2.0×10^{-8}	3.2×10^{-8}	13.50[e]	nd
	5.0×10^{-8}	3.1×10^{-8}	20[f]	92
PAN	5.0×10^{-8}	3.1×10^{-8}	>25[f]	84
TAN	5.0×10^{-8}	3.1×10^{-8}	>25[f]	92
2,9-dmp	5.0×10^{-8}	3.1×10^{-8}	>25[f]	91

[a]abbreviations: 2,9-dmp = 2,9-dimethyl-1,10-phenanthroline; TAN = 1-(2-thiazolylazo)-2-naphthol.

[b]mol L^{-1}.

[c]relative to free Cu(II) = 1.00.

[d]20% n-butanol in n-hexane.

[e]Ahsanullah & Florence (1984).

[f]Florence et al. (1984).

nd = not determined.

A high toxicity index implies that the complex is more toxic than inorganic aqueous copper(II). Lipid solubility and strong chelation with copper are essential for high toxicity. For example, copper(II) complexes of oxine, PAN and TAN were exceptionally toxic to *Nitzschia closterium*, whereas the non-chelating isomers of these ligands (4-hydroxyquinoline, 1-(3-pyridylazo)-2-naphthol), and 1-(3-thiazolylazo)-2-naphthol) did not enhance copper toxicity (Florence et al., 1984). Further, addition of substituents to a ligand which increase its water solubility substantially reduce its toxic effect with copper(II). For example, the copper(II) complex of bathocuproine had a toxicity index of 2.5, whereas that for its disulphonate derivative was <0.1 (Florence et al., 1984).

Mechanism of Toxicity

Lipid-soluble copper complexes can diffuse directly through cell membranes; thus, both the metal and the ligand enter the cell. The ligand may be more toxic than the metal ion and/or the metal and ligand may exert synergic toxicity. Various mechanisms for the toxic effects of hydrophobic metal complexes have been proposed (Florence et al., 1984), *viz:* (i) catalysis of hydrogen peroxide and oxygen free radical formation from molecular oxygen; (ii) intercalation of the complexes with DNA; and, (iii) inhibition of DNA or RNA polymerase. Toxicity is not dependent on the oxidation state of copper in the hydrophobic complex. Further, the toxicity exerted by a particular complex is dependent on the particular organism studied. For example, Cu-PAN was extremely toxic to *Nitzschia closterium* but was less toxic to *Chlorella pyrenoidosa* (Stauber & Florence, 1987). With *Nitzschia closterium* the main toxic effect of copper ions was their action within the cytosol, where they may lower the intracellular thiol concentration and inhibit cell division, but not affect other cellular functions (Stauber & Florence, 1986, 1987).

The copper(I) complex of 2,9-dimethyl-1,10-phenanthroline was extremely toxic to *Nitzschia closterium* due to its reaction with H_2O_2 within cells and the production of damaging oxygen free radicals (Florence et al., 1985). It was not determined whether the ligand itself was retained by the algal cells or discharged in an altered form. In contrast, the copper(II) complexes of oxine and PAN are unlikely catalysts for redox reactions (Florence et al., 1984).

It has been reported that copper(II) oxinate is almost completely absorbed by *Nitzschia closterium* cells; the complex dissociates within the cells, copper(II) is retained and oxine is expelled in an unaltered form. The toxicity of this complex was related to the total percentage of copper(II) complexed by oxine and not to the ratio of 1:1 and 1:2 complexes (Florence & Stauber, 1986). In addition to decreasing the cell division rate of *Nitzschia closterium*, oxine and Cu-oxine also depressed the rate of photosynthesis. In contrast, a range of other ligands and their copper complexes (including PAN) reduced the cell division rate but had no effect on photosynthesis (Stauber & Florence, 1987). Data presented by Stauber and Florence (1987) suggested that, in addition to the acute toxicity of the copper complexes of oxine and PAN, these ligands were themselves toxic to *Nitzschia closterium.* (The present work does not support this result for oxine.)

In this work, algal assays have been used to study the potential ameliorating effect of humic substances on the toxicity of lipid-soluble copper complexes to algae. Two hydrophobic metal complexes were used in this work; Cu-(1-(2-pyridylazo)-2-naphthol)$_2$, (Cu-PAN) and Cu-(8-hydroxyquinoline)$_2$, (Cu-oxine). These complexes have large stability constants;

Cu-oxine: log K_1=12.10, log K_2 = 10.90 (Fresco & Freiser, 1964).

Cu-PAN: log K_1=12.6 (Betteridge et al., 1963).

They are also extremely toxic to algae (Florence & Stauber, 1986).

This chapter is divided into 2 sections. In Section A, the use of algal assays to assess the posssible impact of humic substances on the toxicity of hydrophobic copper complexes is described. These assays indicated that there is an interaction between humic substances and the metal complexes, but were unable to establish whether this was of a hydrophilic or a hydrophobic nature. Therefore it was necessary to use other techniques to elucidate this. Specifically, visible spectroscopy and anodic stripping voltammetry (at a Nafion-coated thin-mercury film electrode) were used. Equilibrium dialysis was also used in this study but was found to have inadequate sensitivity; these experiments are briefly discussed in Section B.

SECTION A: ALGAL ASSAYS, DC-ASV, AND SPECTROPHOTOMETRIC STUDIES

8.2 EXPERIMENTAL

8.2.1 Seawater

Surface seawater was collected (on 23 February 1989) 2 km offshore in Bate Bay, Port Hacking, Sydney, Australia, in polyethylene bottles and immediately filtered through a 0.45 µm membrane. Filtered seawater was stored at 4°C; it was equilibrated at room temperature before use in algal assays.

To determine the total concentration of copper in the sample, 25 mL of seawater + 10 µL Suprapur H_2O_2 + 83 µL Aristar HNO_3 was irradiated for 2 h with a 500 W UV lamp. The concentration of copper in the resultant solution was found to be 2.54 x 10^{-8} mol L^{-1} (by

differential pulse ASV measurements at a hanging mercury drop electrode). This analysis was performed by Dr H.K.J. Powell at CSIRO, Lucas Heights, Sydney. Earlier studies have reported the total copper concentration in surface Pacific water off the coast of Sydney to be 0.30 to 0.80 $\mu g\ L^{-1}$ (*ca.* 0.47 to 1.3 x 10^{-8} mol L^{-1}) (Batley & Gardner, 1978). The total organic carbon (TOC) content of the filtered seawater was 2.5 ppm. The TOC analysis was performed by Dr R. Matthews (CSIRO, Lucas Heights, Sydney); details of the method have been reported elsewhere (Matthews et al., 1990).

8.2.2 Algal Cultures

Nitzschia closterium (Ehrenberg) W. Smith, a marine diatom (originally obtained from CSIRO Division of Fisheries Algal Culture Collection, Hobart, Australia) was cultured in f medium (Guillard & Ryther, 1962) except that ferric citrate/citric acid (4.5 mg L^{-1} ferric citrate and 4.5 mg L^{-1} citric acid) was replaced with iron-EDTA, and all other trace metal concentrations were halved.

Stock algal cultures were illuminated at 6 400 lux (fluorescent daylight tubes) on a 12 h light/dark cycle at 21 ± 2°C. Transfers were made under axenic conditions (using autoclaved culture medium, in a laminar flow hood in a class 100 clean room) every 2 weeks. Stock cultures 10 days after transfer (exponential growth phase) were used for algal assays.

8.2.3 Algal Assays

To minimize possible complexation of copper (II) by components of the culture medium, algae were washed 3 times with unsupplemented seawater before use (by centrifugation at 2 500 rpm for 8 min using a JOUAN CR 4 11 centrifuge). The resulting washed algal suspension was homogenized (to disperse clumps) and vortexed to ensure homogeneity. Aliquots of algae were added to 50 mL seawater in silanized 200 mL conical flasks (which had been acetone rinsed and acid washed (10% HNO_3)) to give an initial cell density of 2 x 10^4 to 4 x 10^4 cells mL^{-1}. The flasks were covered with a loose glass cap and illuminated on a light box at 16 000 lux (fluorescent daylight tubes) on a 12 h light/dark cycle at 21 ± 2°C. The higher intensity light used for the assays was to ensure algal division each day.

The density of live cells was measured daily over 4 days by counting microscopically on a haemocytometer (Olympus BH-2 phase contrast microscope, 250 x magnification). Logarithmic growth was maintained over this period. All assays involved replicate counts (at least 4) on duplicate flasks. A blank, consisting of algae in unsupplemented seawater, was monitored for each assay.

8.2.4 Hydrophobic Metal Complexes

To counter possible complications from slow coordination reactions occurring in seawater (Hering & Morel, 1989), and to ensure an identical metal:ligand ratio in each flask, the copper(II)-ligand complex (CuL) was pre-formed in Analar ethanol. Addition of 50 µL of the stock ethanolic CuL solution to 50 mL of seawater generated 3.1 x 10^{-8} mol L^{-1} Cu(II) and 6.2 x 10^{-8} mol L^{-1} ligand in the final assay solution. Each flask was prepared in duplicate.

PAN (1-(2-pyridylazo)-2-naphthol) was obtained from Sigma and recrystallized from 'Spectroscopic' ethanol (BDH) before use. Microanalysis established the composition: C, 72.15%; N, 16.94%; and H, 4.62% (c.f. calc. for $C_{15}H_{11}N_3O$: C, 72.21%; N, 16.86%; and H, 4.41%). Oxine (8-hydroxyquinoline), BDH Analar, was used without purification. Stock ligand solutions were prepared in ethanol and stored in the dark.

In assays involving humic substances, CuL and the humic or fulvic acid were allowed to equilibrate in seawater for at least 2 h before addition of the algal suspension (unless otherwise stated).

8.2.5 Determination of K_{ow}

The K_{ow} value for a compound is determined by partitioning the compound between octanol and water. The K_{ow} value for Cu-oxine and Cu-PAN was determined by equilibrating a solution (10 mL) of 2 x 10^{-6} mol L^{-1} Cu(II) and 6 x 10^{-6} mol L^{-1} ligand in 10^{-3} mol L^{-1} acetate buffer (pH 5.5) with 10 mL octan-1-ol; (the solutions were mixed for 12 h). The organic phase was then washed several times with Milli-Q water to remove any colloidal material, followed by extraction with 0.1 mol L^{-1} HNO_3 to release Cu(II) from the octanol. The copper content of the acid extract was measured by DC-ASV at a HMDE; this corresponded to the concentration of CuL

in the octanol phase. The concentration of CuL in the aqueous phase was then obtained by the difference between the amount of Cu(II) added to the solution (2×10^{-6} mol L^{-1}) and the concentration of Cu(II) in octanol. All experiments were performed in a class 100 clean room.

8.2.6 Humic Substances

Algal assays were performed using two different concentrations of humic substances; 1 ppm and 5 ppm. An International Humic Substances Society (IHSS) reference soil humic acid, Summit Hill humic acid (SHHA), was used. A stock solution of SHHA in seawater was prepared (5 ppm). SHHA was very sparingly soluble in seawater (probably due to the high concentration of calcium ions). Therefore, to effect dissolution, the SHHA was "predissolved" in 200 µL of 0.8 mol L^{-1} NaOH, stood for approximately 1 h, followed by dilution to 500 mL with seawater; immediately after this dilution, HCl was added to adjust the pH to 8.0 (the pH of the seawater sample). Some precipitation of SHHA was observed in the 5 ppm stock solution over the time scale of the assays (4 days).

Fulvic acid was completely soluble in seawater (no material was retained on a 0.025 µm membrane). A stock solution of fulvic acid in Milli-Q water was prepared (1 000 ppm); aliquots of this solution were added to each flask to generate the 1 ppm and 5 ppm solutions.

8.2.7 Anodic Stripping Voltammetry

ASV measurements were performed with laboratory built glassy carbon, reference (Ag,AgCl in 1 mol L^{-1} KCl, with vycor junction), and counter (Pt) electrodes coupled to a PAR 174 potentiostat and a PAR RE0074 X-Y recorder. Instrumental parameters were: nitrogen flush, 10 min; mode, DC; scan rate, 100 mV s^{-1}; deposition potential, -0.60 V; rate of stirring of solution, *ca.* 700 rpm (Teflon-coated magnetic stirrer bar); deposition time, 5 min (unless otherwise stated). All experiments were carried out in a class 100 clean room.

A stock solution of Tris buffer (pH 8.2) was prepared by mixing appropriate volumes of Tris (Fluka, puriss p.a.) and Tris.HCl (Koch-Light, puriss); this solution was electrolytically purified by electrolysis at a mercury pool cathode at -1.0 V. Each solution was prepared in 0.01 mol L^{-1} Tris buffer, pH 8.2, containing 0.1 mol L^{-1} KNO_3 (unless otherwise stated). The total

volume in the cell was *ca.* 5 mL. Aliquots of stock PAN (in 'Spectroscopic' ethanol) and copper(II) solutions were added (*via* a Volac micropipette) to generate the required concentrations.

Preparation of the Nafion-coated thin-mercury film electrode (NCTMFE) is described in Chapter 7.

8.2.8 Spectrophotometric Measurements

Spectrophotometric measurements were made on a Varian Superscan 3 spectrophotometer, using matched 50 mm quartz cells. The effect of humic substances on the spectra of 1:1 and 1:2 Cu-PAN complexes (3×10^{-7} mol L^{-1} Cu(II), 6×10^{-7} mol L^{-1} PAN) was measured 'by difference' (0 - 50 ppm humic substance in the test and reference cells). All solutions were prepared in 10^{-2} mol L^{-1} Tris buffer (pH 8.2). The SHHA was 0.025 μm filtered before use (filtration was effected at pH *ca.* 7.0 following dissolution of the humic acid at high pH).

8.3 RESULTS

8.3.1 Properties of the Hydrophobic Metal Complexes Studied

Although, to the author's knowledge, no log K_2 value for Cu-PAN has been reported in the literature, the 1:2 stoichiometry of the PAN and oxine copper(II) complexes was established by UV-visible absorption spectrophotometry. Addition of oxine (6×10^{-7} mol L^{-1}) to a solution of Cu-PAN ([Cu(II)] = 3×10^{-7} mol L^{-1}; [PAN] = 6×10^{-7} mol L^{-1}) at pH 8.2 resulted in a 70% decrease in the Cu-PAN absorption peak (at 550nm); this establishes that the effective (pH-dependent) stability constant for the bis-oxinato complex is greater than that for the 1:2 complex. This observation was supported by calculations with the equilibrium program SIAS (including the hydroxy species $CuOH^+$, log β = -7.71 and $Cu_2(OH)_2$, log β = -10.99 (Sylva & Davidson, 1979) (*vide infra*). If log K_1 for Cu-PAN was assumed to be 12.60 (Betteridge et al., 1963) then the SIAS calculations predicted 98.5% displacement of PAN in a solution 6×10^{-7} mol L^{-1} in ligand and 3×10^{-7} mol L^{-1} in Cu(II) at pH 8.2; or 74% displacement based on log K_1 = 16 (Pease & Williams, 1959).

The hydrophobicities measured for Cu-PAN and Cu-oxine were similar: log K_{ow} for Cu-PAN was 0.46 while that for Cu-oxine was 1.70. These results (expressed as percentage complex extracted from water) are comparable to those reported by Florence and coworkers; Table 8.2.

Table 8.2: Percentage Copper(II) Complex Extractable From Water

	20% n-butanol in n-hexane[a]	n-octanol
Cu-oxine	92	78[b], 97[c]
Cu-PAN	84	84[b], 72[c]

[a]Florence et al., 1984 ([Cu(II)] = 3.15×10^{-8} mol L^{-1}; [ligand] = 5×10^{-8} mol L^{-1}).

[b]Florence & Stauber, 1986 ([Cu(II)] = 3.15×10^{-8} mol L^{-1}; [ligand] = 5×10^{-8} mol L^{-1}).

[c]This work.

Sep-Pak C_{18} catridges have been widely used to concentrate humic substances, hydrophobic organic substrates, and nonpolar metal complexes from natural waters (Mills & Quinn, 1981; Mackey, 1983; Donat et al., 1986; Junk & Richard, 1988; Zhou & Wangersky, 1989; Amador et al., 1990). When 25 mL of Cu-PAN and Cu-oxine solutions (3×10^{-7} mol L^{-1} Cu(II); 6×10^{-7} mol L^{-1} ligand) were passed through 200 mg Activon Extra-Sep C_{18} cartridges about 40% of the copper(II) complex was retained in each case.

8.3.2 Algal Assays

Humic Acid and Cu-Oxine

For these assays the humic acid and Cu-oxine were equilibrated for approximately 30 min in the seawater culture medium before addition of algae. Results are summarized in Table 8.3. The presence of Cu-oxine resulted in cell death within 24 h. With 1 ppm SHHA and Cu-oxine some initial cell death occurred during the first 24 h, followed by some cell division by the remaining algae. On leaving the 1 ppm SHHA and Cu-oxine to equilibrate in seawater for 2 h

prior to addition of algae, cell numbers remained constant over 4 days; Table 8.4. Based on this observation, humic substances and the copper complex were equilibrated for 2 h before addition of algae in all subsequent experiments.

The toxicity of Cu-oxine was completely ameliorated in the presence of 5 ppm SHHA with normal cell division observed, i.e. the growth rate was the same as that for the blank (seawater only). However, with 0.025 μm filtered 5 ppm SHHA (resulting in a concentration of *ca.* 1.5 ppm, *vide infra*) cell numbers remained constant in the presence of Cu-oxine (as observed with 1 ppm unfiltered SHHA); Table 8.4. Typical growth curve plots are shown in Figure 8.1.

Table 8.3: The Effect of Humic Acid on the Toxicity of Cu-oxine to *Nitzschia closterium*

Solution		Day 1	Av	Day 2	Av	Day 3	Av
Blank	(i)[a]	10,12,6,18	12	45,38,32,24	35	26,22,29,43,31, 24	30
	(ii)	14,11,10,19	14	35,26,10,17	22	52,47,32,47	45
1 ppm SHHA	(i)	15,5,19,11	13	37,32,37,28	34	50,53,39,46	47
	(ii)	16,22,13,12	16	45,5,11,29,26,48	28	34,51,36,38	40
Cu-oxine	(i)	4,2,3,2	3	2,1,3,1	2	2,0,0,1	1
	(ii)	3,1,2,3	3	0,1,0,0	0	1,3,3,3	3
1 ppm SHHA + Cu-oxine	(i)	0,1,4,3	2	11,12,10,8	11	8,12,12,20	13
	(ii)	2,3,5,3	4	7,14,14,14	13	17,19,16,17	18
5 ppm SHHA	(i)	13,17,16,22	17	20,36,32,50,21, 39	33	48,57,48,51	51
	(ii)	12,14,16,26	16	17,22,17,19	19	20,22,18,14,16	18
5 ppm SHHA + Cu-oxine	(i)	20,12,12,26	18	28,28,19,28	26	54,52,36,39,53, 55	49
	(ii)	18,17,13,8,9, 15	14	28,24,31,34	30	40,43,34,18,36, 43	36

[a] In all experiments (i) and (ii) refer to duplicate assays with the same solution composition.

Table 8.4: The Effect of Humic Acid on the Toxicity of Cu-oxine - Influence of Equilibration Time and Use of Filtered Humic Acid

Solution		Day 1	Av	Day 2	Av	Day 3	Av
Blank	(i)	18,11,16,15	15	23,42,25,47,35,35	35	50,71,29,37,46,55	48
	(ii)	16,13,14	15	38,35,33,37	36	31,27,37,37	33
Cu-oxine	(i)	10,9,7,5	8	1,1,0,0	0	0,0,0,0	0
	(ii)	5,7,6,7	7	0,1,0,1	0	0,0,0,0	0
1 ppm SHHA + Cu-oxine[a]	(i)	24,18,17,12	18	9,28,8,11,17,25	17	11,5,14,14	11
	(ii)	6,13,12,9,9,13	11	20,20,13,20	19	13,18,13,11	14
5 ppm SHHA[b] + Cu-oxine	(i)	12,22,8,10	13	8,3,10,5	7	8,10,7,7	8
	(ii)	16,22,6,11,7,14	13	13,11,11,10	12	15,18,14,17	16

[a]SHHA and Cu-oxine equilibrated for 2 h before addition of algae. [b]0.025 μm filtered SHHA.

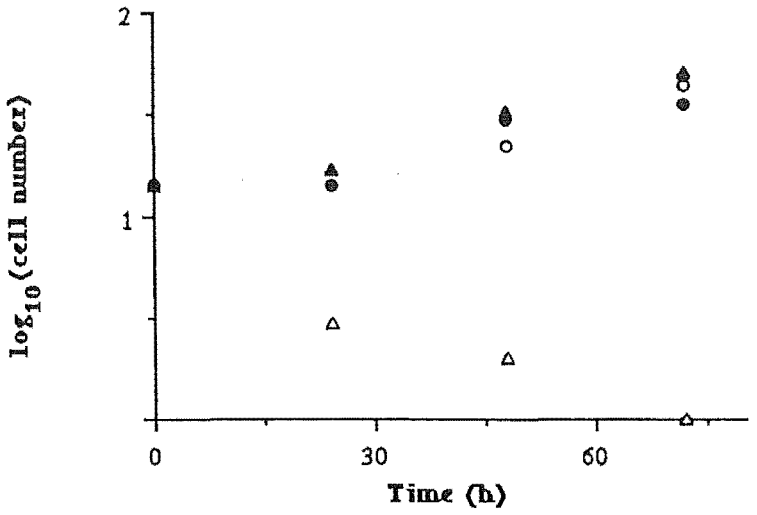


Figure 8.1: Growth Curve Plots for *Nitzschia closterium:* Effect of Humic Acid and Cu-oxine

Assays conducted in seawater containing: o, no other additives; ▲, 5 ppm SHHA; ●, 5 ppm SHHA + Cu-oxine (3.1×10^{-8} M Cu(II), 6.2×10^{-8} M oxine); Δ, Cu-oxine

Fulvic Acid and Cu-Oxine

Cu-oxine caused cell death; 1 ppm fulvic acid (FA4) did not ameliorate this toxicity (Table 8.5). With 5 ppm FA4 and Cu-oxine cell division was inhibited and cell numbers remained constant for the duration of the assay.

Table 8.5: The Effect of Fulvic Acid on the Toxicity of Cu-oxine to *Nitzschia closterium*

Solution	Day 1	Av	Day 2	Av	Day 3	Av
Blank (i)	20,17,20,24	21	22,26,32,36	29	42,57,52,42	49
(ii)	15,11,7,16,15, 10	13	38,32,30,31	33	40,30,25,30,64,45	39
1 ppm FA4 (i)	15,13,11,12	13	35,45,25,21,32,27	31	47,44,48,57	49
(ii)	14,19,21,10,11,13	15	40,36,11,29,23,36	30	33,19,59,51,48,56	45
Cu-oxine (i)	7,5,6,5,3	6	4,4,3,2	4	0,0,1,1	0
(ii)	7,5,3,7	6	0,0,0,0	0	0,0,0,0	0
1 ppm FA4 + Cu-oxine (i)	10,7,3,4,14,9	8	3,4,2,6	4	0,3,2,3	2
(ii)	5,8,7,11	8	3,0,2,5	3	1,0,3,0	2
5 ppm FA4 (i)	17,18,15,16	17	42,41,22,29	34	30,37,46,58,38,29	40
(ii)	7,12,18,11,11,18	13	36,32,27,27	31	47,53,42,27	43
5 ppm FA4 + Cu-oxine (i)	13,7,11,11	11	12,13,8,10	11	18,14,8,12,7,5	11
(ii)	5,13,13,10,11, 12	13	11,18,15,9	14	6,9,9,5	8

Humic and Fulvic Acids and Cu-PAN

It was observed that Cu-PAN is extremely toxic to *Nitzschia closterium;* all cells died within 24 h of being exposed to this complex (Table 8.6). Cell death also occurred in the presence of 1 ppm SHHA or 5 ppm FA4; in contrast, cell numbers remained constant with 5 ppm SHHA.

Table 8.6: The Effect of Humic and Fulvic Acids on the Toxicity of Cu-PAN to *Nitzschia closterium*

Solution	Day 1	Av	Day 2	Av.	Day 3	Av.
Blank (i)	18,34,20,16,13,18	20	61,48,37,71,51,74	57	89,92,72,89	86
Blank (ii)	21,20,14,31,21,22	22	58,58,50,61	57	66,102,87,93	87
1 ppm SHHA (i)	33,23,27,21	26	62,49,51,54	54	89,115,126,86,70,89	96
1 ppm SHHA (ii)	19,25,21,25	23	81,67,69,63	70	81,65,114,96, 80	88
Cu-PAN (i)	0,1,1,0	0	0,0,0,1	0	0,0,0,0	0
Cu-PAN (ii)	0,1,0,0	0	0,0,0,0	0	0,0,0,0	0
1 ppm SHHA + Cu-PAN (i)	2,0,1,1	1	2,0,0,0	0	0,0,0,0	0
1 ppm SHHA + Cu-PAN (ii)	0,1,0,0	0	0,0,0,0	0	0,0,0,0	0
5 ppm SHHA (i)	23,31,33,35,23,22	28	50,76,45,35	52	119,155,109, 116	125
5 ppm SHHA (ii)	39,35,33,27	34	44,38,77,57, 61,77	59	132,141,90,63,92, 110	105
5 ppm SHHA + Cu-PAN (i)	16,20,7,15,21, 20	17	9,10,9,7	9	12,14,12,13	13
5 ppm SHHA + Cu-PAN (ii)	14,26,10,10,21,17	17	11,9,8,5,9,8	9	10,5,2,7,7,3	7
5 ppm FA4 + Cu-PAN (i)	1,0,0,0	0	0,0,0,0	0	0,0,0,0	0
5 ppm FA4 + Cu-PAN (ii)	0,0,0,0	0	0,0,0,0	0	0,0,0,0	0

To investigate this latter result, a longer pre-equilibration time was tried, with the 5 ppm SHHA/Cu-PAN /seawater solution being left for about 60 h before addition of algae. However, assay results were not reproducible (6 replicates); Table 8.7. In some cases cell numbers remained constant, while in others cell death occurred.

Table 8.7: Effect of HA-CuPAN Equilibration Time on Amelioration of Toxicity

Solution	Day 1	Av.	Day 2	Av.	Day 3	Av.
(i) Blank	16,14,18,28	19	33,29,35,19,28	29	31,22,41,31,43	34
(ii)	19,22,17	19	25,29,25,32	28	40,35,36,44,31	37
(i)	7,10,16,8	11	1,2,0,1	1	0,0,2,0	0
(ii) 5 ppm	13,13,22,27,19,18	17	38,36,37,38	38	30,12,28,45,43,43	34
(iii) SHHA +	3,4,2,2,	3	1,1,0,0	1	0,0,0,0	0
(iv) Cu-PAN	18,15,19,15	17	17,21,30,25	23	25,31,27,26	27
(v)	5,7,5,5,	6	1,2,0,0	1	0,0,0,0	0
(vi)	15,11,15,12	13	21,20,21,14	19	19,17,18	18

Toxicity of PAN and Oxine

To measure the toxicity of the ligands themselves, assays were conducted in media containing oxine or PAN with no added copper(II). Normal growth was observed in the presence of 6.1×10^{-8} mol L^{-1} and 1.2×10^{-7} mol L^{-1} oxine, and also with 6.1×10^{-8} mol L^{-1} oxine in the presence of 5 ppm SHHA.

Consistent with the earlier study (Florence & Stauber, 1986), cell death occurred in the presence of 3.1×10^{-8} mol L^{-1} PAN; Table 8.8.

Table 8.8: Toxicity of PAN and Oxine (with no added Cu(II))

Solution	Day 1	Av.	Day 2	Av.	Day 3	Av.
Blank (i)	17,20,13,25	19	59,56,61,64	60	52,54,76,61	61
(ii)	20,27,17,20	21	49,38,43,40	43	45,61,70,63	60
PAN[a]	9,8,8,10	9	1,1,0,1	1	0,0,0,0	0
PAN[b]	10,12,8,6	9	0,0,1,0	0	0,0	0
oxine[c*] (i)	16,8,8,19,20,11	14	24,35,15,21	24	51,66,44,45	52
(ii)	13,16,13,13	14	22,24,26,29	26	39,21,43,59	41
5 ppm SHHA + oxine[c*(ii)] (i)	16,19,23,8	17	46,45,52,60	51	81,70,72,82	77
	21,21,13,12	17	25,32,36,39	33	30,49,61,69, 40,43	49
oxine[d*] (i)	30,25,7,19,18, 16	20	18,13,43,31,23,21	25	22,41,42,41	37
(ii)	21,5,11,9,5,17	12	25,16,40,33	29	37,37,36,46	39

[a] 3.1×10^{-8} mol L^{-1} PAN.

[b] 6.2×10^{-8} mol L^{-1} PAN.

[c] 6.1×10^{-8} mol L^{-1}.

[d] 1.2×10^{-7} mol L^{-1}.

* Blank of Table 4 applies to these assays.

8.4 DISCUSSION

8.4.1 Growth Medium

Many assay procedures are carried out in f medium (Guillard & Ryther, 1962); however, this contains chelators and adsorbents which may bind the metal ions being tested for toxicity. This problem was overcome by culturing *Nitzschia closterium* in a modified f medium then conducting assays in raw unenriched seawater (Stauber & Florence, 1985a,b, 1986, 1987). Unsupplemented seawater can support the logarithmic growth of *Nitzschia closterium* for at least 72 h (Lumsden & Florence, 1983).

Both the composition of the assay medium and the maintenance culture used before the assay are critical in determining metal toxicity (Millington et al., 1988). On comparison of copper and zinc toxicity to *Nitzschia closterium* in f medium and unsupplemented seawater, Stauber and Florence (1989) recommended that unenriched seawater be used for marine algal assays.

In all assays, an initial lag in growth was observed with no significant cell division occurring within 24 h. That is, the cell numbers on "Day 1" were the same as the initial cell density. It is possible that the algae required a certain time to recover from the assay preparation procedure (washing, centrifugation, vortexing, homogenizing, and being placed in a new culture medium) before cell division could resume. Alternatively, the harshness of the sample preparation technique may have resulted in not all algae being able to divide.

8.4.2 Concentration of Humic Substances and Metal Complexes

The concentrations of humic substances used in this work (1 ppm and 5 ppm) are environmentally significant. According to Thurman (1985), rivers and lakes contain approximately 2 to 10 mg L^{-1} dissolved organic carbon (DOC), ground waters 0.7 mg L^{-1}, and seawater 0.5 mg L^{-1}. The DOC concentration of 0.5 mg L^{-1} in seawater is equivalent to approximately 1 mg L^{-1} (1 ppm) humic substance. This value corresponds to the lower concentration used in this work. The higher humic substance concentration used (5 ppm) may be pertinent in near shore and harbour waters, and in estuarine environments. The concentration of DOC is greatest in surface seawater and decreases rapidly with depth (Marty et al., 1988; Sugimura & Suzuki, 1988).

The copper(II) and ligand concentrations employed in this study were chosen to be comparable with previous work (Florence et al., 1984; Florence & Stauber, 1986); these values are also environmentally relevant.

It is possible that if there are some very strong copper(II) complexing functional groups in humic substances, then some displacement of the ligand in the hydrophobic copper(II) complex by humic moieties may occur. This would be dependent on the relative stability constants of the humic moieties and the hydrophobic copper(II) complex. The

equivalent weight (weight per mole of COOH) of the fulvic acid sample is 139 (Gregor, 1987). Assuming (as a maximum) that 18% of the carboxyl groups are citrate moieties (Gregor, Powell & Town, 1989a,b), then at 5 ppm fulvic acid the ratio [citrate]:[Cu(II)] is 72:1. Calculations with the chemical equilibrium program SIAS, using ligands which have been proposed as models for fulvic acid (Gregor, Powell & Town, 1989a,b), indicated that citric acid and salicylic acid (at concentrations applicable to the algal assay conditions) would not be able to displace PAN or oxine from copper(II). However, the SIAS calculations and spectrophotometric measurements indicated that a low concentration of a moiety with a very strong affinity for Cu(II), e.g. 3×10^{-6} mol L^{-1} histidine (an amino acid residue which has been characterized in humic substances; Schnitzer, 1985), can cause some displacement of PAN from Cu-PAN. According to the SIAS calculations, this concentration of histidine would completely displace PAN from Cu(II); however, only a 14 % reduction in Cu-PAN absorbance at 550 nm was observed by visible spectroscopy. (This concentration of histidine is equivalent to *ca.* 50% of the typical nitrogen content of humic substances). Interestingly, Mills et al. (1987) reported that less than 1% of the total acidic sites in organic matter were involved in copper complexation in estuarine waters.

8.4.3 Chemical Modelling of Copper Complexes in Seawater

The possible species distribution of copper complexes in seawater was calculated by use of the chemical equilibrium program SIAS (Fardy & Sylva, 1978). Calculations were performed for the algal assay experimental conditions (3.1×10^{-8} mol L^{-1} Cu(II), 6.2×10^{-8} mol L^{-1} ligand (PAN or oxine), 0.012 mol L^{-1} Ca(II), 0.0532 mol L^{-1} Mg(II), 0.536 mol L^{-1} chloride, 0.0276 mol L^{-1} sulphate, 0.00232 mol L^{-1} CO_3^{2-} and included the complexes likely to be present in seawater (Table 8.9).

Table 8.9: Stability Constants For Inorganic Complexes in Seawater

Complex	log β	Complex	log β
$CaSO_4$	1.00	HCO_3	10.00
$CaCO_3$	3.20	H_2CO_3	16.00
$CaHCO_3$	11.50	$CuSO_4$	2.28
CaOH	-12.70	CuCl	0.20
$MgSO_4$	1.00	CuOH	-7.71
$MgCO_3$	2.20	$Cu_2(OH)_2$	-10.99
$MgHCO_3$	12.40	$Cu(CO_3)_2$	9.80
MgOH	-11.40	$Cu(HCO_3)_2$	5.90
		$CuHCO_3$	3.50

The stability constants included in the SIAS calculations for PAN and oxine species are given in Table 8.10. No stability constant data were available for the Ca(II) and Mg(II) PAN complexes.

Table 8.10: Stability Constants for PAN and Oxine Complexes

Complex	log β	Complex	log β
HPAN	12.20	Hoxine	9.81
H_2PAN	14.10	H_2oxine	14.72
Cu-PAN	12.60	Cu-oxine	12.10
		Cu-$(oxine)_2$	23.00
		Ca-oxine	4.40
		Ca-$(oxine)_2$	8.00
		Mg-oxine	4.40
		Mg-$(oxine)_2$	8.58

The calculated distribution of copper complexes in seawater, using these tabulated data, are given in Tables 8.11 and 8.12.

Table 8.11: Distribution of Copper in Seawater in the Presence of PAN

Complex	Percentage of Total Copper
Cu-PAN	66.2
$CuSO_4$	13.8
CuCl	3.4
CuOH	12.4
free Cu	4.0

Table 12: Distribution of Copper in Seawater in the Presence of Oxine

Complex	Percentage of Total Copper
Cu-oxine	37.1
$Cu\text{-}(oxine)_2$	47.6
$CuSO_4$	6.3
CuCl	1.6
CuOH	5.6
free Cu	1.8

The calculated percent formation of Cu-oxine reported by Florence and Stauber (1986) was 84%, which is the sum of the 1:1 and 1:2 complexes calculated in the present work. It is unclear whether the value reported by Florence and Stauber (1986) referred to

the 1:2 complex or the sum of the 1:1 and 1:2 Cu-oxine complexes. However, this fact may not be critical because, as noted above, the toxicity of Cu-oxine to *Nitzschia closterium* was found to be dependent on the total percentage of Cu(II) complexed by oxine, and not on the ratio of the 1:1 and 1:2 complexes (Florence and Stauber, 1986).

The calculated percent formation of Cu-PAN may be much less reliable. As noted previously, this work has established that PAN forms a 1:2 complex with Cu(II), yet to the author's knowledge no stability constant has been published for this species. Further, the stability constants reported for the 1:1 Cu-PAN complex are not in good agreement, *viz:* log K_1 = 12.60 (Betteridge et al., 1963), or 16 (Pease & Williams, 1959). Since the log K_1 value reported by Betteridge et al., (1963) was measured in 0.1 mol L^{-1} $NaClO_4$, their value was used in the present work. Florence and Stauber (1986) reported 100% formation of Cu-PAN in seawater; it is assumed that they used log K_1 = 16 in their calculations (this value was measured in 20% dioxan). If log K_2 is assumed to be 10.0 (based on comparison with similar ligands) then SIAS calculations indicated 64% formation of the 1:1 Cu-PAN complex and 26% formation of the 1:2 complex. In any case, calculations indicate that there should be significant formation of Cu-oxine and Cu-PAN under the experimental conditions.

In addition to the problem of obtaining reliable stability constants for a particular complex, there are problems associated with using stability constant data to calculate species distributions outside the pH range and concentrations under which the constants were determined (see Chapter 9). Further, published values for metal complex stability constants can vary over a wide range; this is especially so for chloro-complexes. As seawater contains a high concentration of chloride, these stability constants could have a significant effect on the calculated species distribution. There are other problems also, such as the lack of data on the presence and/or stability of polymeric species, as well as the possible presence of unidentified chelating agents. The lability of each species is also important (Florence & Batley, 1976). Hence, these calculated distributions should be viewed with caution and taken as only an approximate guide.

8.4.4 Processes for Amelioration of Copper Toxicity

It is of environmental interest to study the toxic forms of metals and how this toxicity may be ameliorated in a natural system. Organisms have evolved mechanisms to maintain low intracellular concentrations of toxic substances (both metals and organic substrates). These strategies include: (i) active expulsion of toxicants after they have entered the cell; (ii) complexation by biologically synthesized ligands; and, (iii) oxidation, reduction, or chemical modification of the xenobiotic, resulting in precipitation, immobilization, or volatilization (Folsom et al., 1986). For example, *Nitzschia closterium* at a cell density of 2×10^4 to 4×10^4 cells mL^{-1} produces an exudate which can complex about 3.1×10^{-7} mol L^{-1} copper(II). This exudate is only produced in response to aqueous copper(II) ions and the amount excreted increases with the concentration of copper(II) (Lumsden & Florence, 1983). However, such exudates cannot detoxify lipid-soluble copper complexes.

Ligands which form stable water-soluble complexes greatly reduce copper toxicity; Table 8.13. Importantly, complexation with humic substances greatly reduces the bioavailability of copper ions.

Copper ion toxicity to *Nitzschia closterium* is also reduced by adsorption of copper on Mn(III) and Fe(III) hydroxide coatings on the cell surface (Stauber & Florence, 1985a,b). These coatings effectively adsorb copper ions and reduce their penetration into cells. Manganese is more effective than iron, possibly because it can also catalytically scavenge toxic superoxide free radicals. The ability of a range of metal ions to protect against copper toxicity to *Nitzschia closterium* was studied by Stauber and Florence (1987), *viz:* Mn(III), Co(III), Al(III), Fe(III), Cr(III), Ni(II), and Zn(II). The trivalent ions were the most effective; indeed, nickel and zinc were unable to reduce copper toxicity. The degree of insolubility of the metal(III) hydroxide provides an indication of its ability to ameliorate copper ion toxicity.

Interestingly, metal hydroxide coatings were unable to inhibit the toxicity of lipid-soluble copper complexes (Stauber & Florence, 1985a,b). Hydrophobic metal complexes may be able to diffuse through hydroxide coatings, and/or the coatings may not be evenly distributed on the cell surface, such that some areas of the biomembrane remain directly

exposed to the external solution. Furthermore, concentrations of inorganic adsorbers such as Fe and Mn oxyhydroxides are usually very low in open-ocean waters (Hirose, 1990).

Thus, to date no method to effect amelioration of the extreme toxicity of lipid-soluble metal complexes has been found. The aim of this work was to investigate the ability of humic and fulvic acids to sequester, and detoxify, hydrophobic copper complexes.

Table 8.13: Toxicity of Water-Soluble Cu(II) Complexes to *Nitzschia closterium*[a]

ligand[b]	[ligand][c]	[Cu(II)][c]	Toxicity Index[d]	Percentage solvent extractable Cu(II)[e]
NTA	2.0×10^{-6}	3.10×10^{-7}	0.20	<10
LAS	2.0×10^{-6}	3.10×10^{-7}	0.25	13
PAR	5.0×10^{-8}	3.10×10^{-8}	<0.1	<10
TAR	5.0×10^{-8}	3.10×10^{-8}	<0.1	<10
tannic acid	5.9×10^{-7}	3.15×10^{-7}	0.13	nd
fulvic acid	1.0×10^{-5}	3.15×10^{-7}	0.08	nd
humic acid	6.4 mg L^{-1}	3.15×10^{-7}	0.70	nd

[a]Florence et al., 1984.

[b] abbreviations: NTA=nitrilotriacetic acid; LAS=linear alkylbenzene sulphonate; PAR=4-(2-pyridylazo)-resorcinol; TAR=4-(2-thiazolylazo)-resorcinol.

[c]mol L^{-1}.

[d]relative to Cu(II) = 1.00.

[e]20%n-butanol in n-hexane.

nd = not determined.

Properties of the Hydrophobic Metal Complexes Studied

The octanol-water partition coefficients for the copper(II) complexes used in this work are orders of magnitude lower than those reported for hydrophobic substrates such as DDT (log K_{ow}= 6.90; Nebeker et al., 1989). Yet, the toxicity of copper to biota is greatly increased in the presence of oxine and PAN. Further, Kruck et al. (1990) reported a K_{ow} value of 0.67 for an uncharged aluminium-maltolate complex; this lipid solubility was sufficient to facilitate transport across cellular membranes even in the absence of an active transport system.

Florence et al. (1984) reported that Cu-PAN and Cu-oxine have very different DP-ASV lability; hence, it was of interest to measure the DC-ASV lability of these complexes. Results from this work are compared in Table 8.14 with those reported by Florence et al.(1984). (The measurements were performed at a hanging mercury drop electrode).

Table 8.14: Percentage Lability of Copper(II) Complexes

	This Work[a]	Florence et al. (1984)[b]
Cu-oxine	100	64
Cu-PAN	75	<0.5

[a] 1 x 10^{-7} mol L^{-1} Cu(II), 2 x 10^{-7} mol L^{-1} ligand, in 0.01 mol L^{-1} Tris/0.6 mol L^{-1} KNO_3.

[b] 3.15 x 10^{-8} mol L^{-1} Cu(II), 5 x 10^{-8} mol L^{-1} ligand, in seawater.

There is obviously a large discrepancy between these results! Several factors may be important. Firstly, direct-current ASV was used in this work, whereas differential-pulse was used by Florence et al. (1984). Differential-pulse measurements depend on the rate of electron transfer reactions on the mercury surface; further, this wave form is particularly prone to adsorption interferences (Gregor & Powell, 1988a; Powell & Florence, 1990). Secondly, Florence et al. (1984) worked in seawater medium and at very low metal and

ligand concentrations. It is possible that Florence was working at a copper(II) concentration close to the complexing capacity of his seawater sample; hence, chelation of Cu(II) by natural organics in seawater may have interfered in the measurements by lowering the mean diffusion coefficient. Waite and Morel (1983) have reported a copper complexing capacity of 2.8×10^{-8} mol L^{-1} for a filtered (0.2 μm) seawater sample.

In this work, the DC-labilities of Cu-oxine and Cu-PAN were unaffected by ionic strength (range 0.01 to 0.6 mol L^{-1} KNO_3). Pseudopolarograms established that these complexes are not directly reduced at a HMDE.

Hence, Cu-PAN and Cu-oxine have a similar degree of hydrophobicity; Cu-oxine is more stable and has slightly greater DC-ASV lability than Cu-PAN.

Amelioration of the Toxicity of Lipid-Soluble Copper Complexes

Three types of response of the algae to the culture medium were observed, *viz:* normal growth; no growth (cell division inhibited); and, decreasing cell numbers (cell death). Fulvic acid was unable to reduce the toxic effect of either hydrophobic complex. The only situation in which complete amelioration of toxicity was observed was with 5 ppm SHHA and Cu-oxine. Humic acid could not completely inhibit Cu-PAN toxicity. Even when the SHHA/Cu-PAN/seawater solution was equilibrated for 60 h before addition of algae the toxicity of Cu-PAN was not completely ameliorated (in some cases cell numbers remained constant as opposed to cell death). Further, in an environmental situation an influx of a hydrophobic species into a natural water would not have this time (60 h) to equilibrate with humic substances before exposure to aquatic organisms.

Algal growth typically has two stationary phases *viz*, an initial stationary phase, followed by a period of exponential growth, then a second stationary phase when cell division decreases. Some toxicants can lengthen the initial stationary phase by their impact on cell physiology; a percentage of the cells will remain active and normal growth will resume when cells are resuspended in fresh nutrient medium (Stauber, pers. comm. 1989). Hence, it is possible that the toxic effect exerted by Cu-PAN was altered in the presence of 5 ppm

SHHA such that inhibition of cell division, rather than cell death, occurred. A similar effect was observed with 1 ppm SHHA and Cu-oxine, and 5ppm FA4 and Cu-oxine.

The toxicity of the ligands themselves is also important. PAN, in the absence of added copper, was extremely toxic to *Nitzschia closterium*. However, oxine itself was not toxic (Table 8.8). This result is in contrast to earlier work in which oxine was observed to be toxic to *Nitzschia closterium* (Stauber & Florence, 1987). However, it is noted that the toxic effect exhibited will be very sensitive to traces of copper in the seawater culture medium, and it is possible that the seawater sample used in the Stauber and Florence (1987) study had a higher copper content than that used in the present work. Further, the observation that oxine is expelled by *Nitzschia closterium* in an unaltered form (Florence and Stauber, 1986) may suggest that this ligand does not exert a toxic effect.

Nutrient Effect of Humic Substances

In general, the growth rate in the presence of humic substances was the same as that in raw seawater; hence, results do not need to be corrected for any effects due to the presence of humic substances.

However, in one experiment, the presence of humic acid did result in an increase in cell division relative to that observed in unsupplemented seawater (5 ppm SHHA, Table 8.6). This may be because a slightly greater initial cell density was used in this particular assay. With more algae present, nutrients in the seawater culture medium would be depleted more rapidly and may become growth limiting. In such a situation, humic substances may be able to provide nutrients.

8.4.5 Interpretations

The observation that humic substances can have an impact on the toxicity of hydrophobic copper complexes has important environmental implications. A variety of mechanisms may be responsible for this ameliorating effect; these are now discussed.

Hydrophobic Dissolution in a Humic Phase

The interaction of humic substances with lipid-soluble compounds, such as DDT, has been described in terms of a hydrophobic partition mechanism which is proposed to be mechanistically similar to the solubilization of nonpolar solutes by micelles (Chiou et al., 1986).

Results from algal assays are inconclusive, providing evidence both for and against this proposal. In support of a hydrophobic partition mechanism is the observation that humic acid was more effective at reducing the toxicity of lipid-soluble copper complexes than was fulvic acid. (Soil derived humic acid has a greater ability to solubilize nonpolar solutes than does fulvic acid (Chapter 1).) It was also observed that 0.025 μm filtration of the 5 ppm SHHA-seawater solution eliminated its ability to inhibit Cu-oxine toxicity. Chiou (pers. comm., 1989) stated that "filtration [membrane] would most likely remove some large-molecular-weight or less soluble humic components which could be far more important in concentrating highly insoluble organic compounds (by partition rather than by surface adsorption)". He further stated that "the filtered fraction probably has a better [hydrophilic] metal-complexing capacity". Lipoidal components are also likely to be retained on the filter; such moieties may predominate in humic-nonpolar substrate interactions (Pierce et al., 1974; Gauthier et al., 1987).

In a partitioning mechanism the algal cells will always act as a competing hydrophobic phase. The biomembrane of these cells may be more hydrophobic than are components of humic substances. The applicability of the log K_{ow} value to these two different hydrophobic phases is unclear.

Algal assays also provided evidence which is *not* consistent with a hydrophobic partitioning mechanism. The copper(II) complexes used in the present work had similar log K_{ow} values; hence, if hydrophobic dissolution was involved then the toxicity of both complexes may have been expected to be ameliorated to a similar extent.

At the concentration of humic substances employed in these studies, formation of humic acid micelles is unlikely. In exploring the micelle model for humic acid proposed by Wershaw (1986), a mean molecular weight of 10 000 Dalton for individual humic acid

molecules and a micelle aggregation number of 100 units has been assumed (Town & Powell, 1989). If humic acid sequestered lipid-soluble metal complexes by dissolution in the hydrophobic core of a micelle then there were barely sufficient humic acid molecules present for such a mechanism to be in effect under algal assay conditions (at 5 ppm SHHA, the micelle:metal complex ratio is 1:6). Further, the humic acid concentrations employed were probably well below any critical micelle concentration. In addition, Cu-PAN and Cu-oxine are much less hydrophobic than compounds such as DDT. Humic acid is reported to be quite a polar partition medium (Chiou et al., 1986) and some workers have found that humic acid has a greater affinity for polar compounds than for hydrophobic substrates (Antworth et al., 1989; Collazo-Lopez et al., 1989).

Adsorption on Colloidal Humic Particles

The observation that filtered (0.025 μm) humic acid could not ameliorate Cu-oxine toxicity may suggest that it is the colloidal/particulate humic components which are involved in interactions with nonpolar compounds. Alternatively, this phenomenon may merely be a concentration effect; thus, for a humic acid solution (14 ppm) prepared in 0.1 mol L^{-1} Tris buffer (pH 8.2) a 27.5% decrease in absorbance at 300 nm was observed following filtration (0.025 μm). For a parallel experiment in seawater a 71% decrease in absorbance was observed. That is, the effective concentration of a filtered 5 ppm SHHA solution may be approximately 1.5 ppm, which may not be sufficient to ameliorate toxicity.

Formation of a Protective Humic Coating on the Surface of Algal Cells

Another possible mechanism for the amelioration of toxicity of hydrophobic copper complexes by humic acid is that humic substances may form a protective coating on the surface of the algal cells which acts as a physical barrier to the xenobiotics. This could be analogous to the formation of metal(III) hydroxide coatings which reduce copper(II) ion toxicity (Stauber & Florence, 1985a,b, 1987).

To test this hypothesis, an aliquot of algae was equilibrated in a 5 ppm SHHA/seawater solution for 2 h. The algae were then filtered (0.6 μm), and rinsed with seawater followed by dilute NaOH (0.02 mol L^{-1}) to dissolve any humic coating. The UV-visible absorption spectrum of the NaOH washings of the humic equilibrated algae, and that of algae which had not been exposed to humic acid, was recorded. No evidence for the presence of a humic coating was obtained; however, this technique may not have been sensitive enough to detect the very small amounts of humic materials involved.

As with the other possible mechanisms, if a protective humic coating is involved, and if it can be assumed that hydrophobic effects are important, then there is no apparent reason why different results should have been observed for Cu-oxine and Cu-PAN.

Hydrophilic Complexation of Copper(II) by Humic Acid

Due to their high affinity for Cu(II) ions, humic substances may complex Cu(II) and displace the hydrophobic ligand, thus detoxifying the lipid-soluble complex by this mechanism. Importantly, complexation of the Cu(II) aqua ion by humic substances (and other water-soluble ligands) did reduce the toxicity of the metal ion to *Nitzschia closterium* (Table 8.13). However, if the free ligand is toxic, as is PAN, then displacement of a coordinated ligand by humic acid will not remove the toxicity. Further, this mechanism requires that humic acid (which has been shown to ameliorate toxicity) complexes Cu(II) more strongly (at pH 8.2) than does fulvic acid.

Hence, the algal assays indicated that humic substances have the potential to reduce the toxic effect exerted by lipid-soluble copper(II) complexes towards *Nitzschia closterium* in seawater medium. However, these studies did not allow the mechanism of this amelioration to be established unequivocally. This finding does, however, have important environmental applications as it may allow the potential toxic impact of metal complexes in soils and natural waters to be predicted. Specifically, this study indicated that the toxicity of a hydrophobic copper(II) complex would be ameliorated if a certain threshold concentration of humic acid is present (5 ppm in seawater) and if the ligand itself is not toxic.

To further elucidate the nature of the interaction between humic substances and hydrophobic complexes other techniques were employed. Specifically, UV-visible spectroscopy and anodic stripping voltammetry were used to probe the interaction of humic and fulvic acids with Cu-PAN and Cu-oxine. These approaches are discussed below.

ASV STUDIES USING THE NAFION-COATED THIN-MERCURY FILM ELECTRODE (NCTMFE)

8.5 INTRODUCTION

Voltammetric techniques can probe the lability of metal species. Interpretation of measurements in the presence of humic substance is confounded by the adsorption of humic moieties on the electrode surface. Recently, a Nafion-coated thin-mercury film electrode has been reported to provide increased resistance to electrode fouling by surface-active substances (Hoyer et al., 1987; Hoyer & Florence, 1987). The present work has established that the adsorption effects of humic substances are eliminated at a NCTMFE (Chapter 7)

Anodic stripping voltammetry at a NCTMFE was used to probe the association of humic substances with Cu-PAN and Cu-oxine. Since Cu-oxine and Cu-PAN are approximately 100% labile at a HMDE (Table 8.14), it was assumed that a measured decrease in the copper(II) peak area in the presence of humic or fulvic acid would indicate association of Cu(II) and/or Cu-L with the humic substance, resulting in a lower concentration of labile metal. Analogous to this, Nelson et al. (1988) measured the association of humic acid with PAHs at a phospholipid-coated HMDE. It was assumed by these authors that "complexation" of PAH by humic acid would result in a decreased voltammetric signal because the humic-bound PAH would be unable to penetrate the lipid coating on the electrode surface.

8.6 RESULTS

8.6.1 Effect of Humic Substances on the Apparent Lability of Cu-PAN

For these experiments, a 1:2 Cu:PAN ratio was used (3 x 10^{-7} mol L^{-1} Cu(II), 6 x 10^{-7} mol L^{-1} PAN) in 0.1 mol L^{-1} KNO_3, 0.01 mol L^{-1} Tris (pH 8.2). The copper(II) peak area was recorded for a range of deposition times (2 to 10 min), then an aliquot of humic substance was added and the experiment was repeated. The percentage supression of Cu(II) peak area by humics was calculated from the relative slopes for plots of peak area *versus* deposition time; Figure 8.2. Results are summarized in Table 8.15.

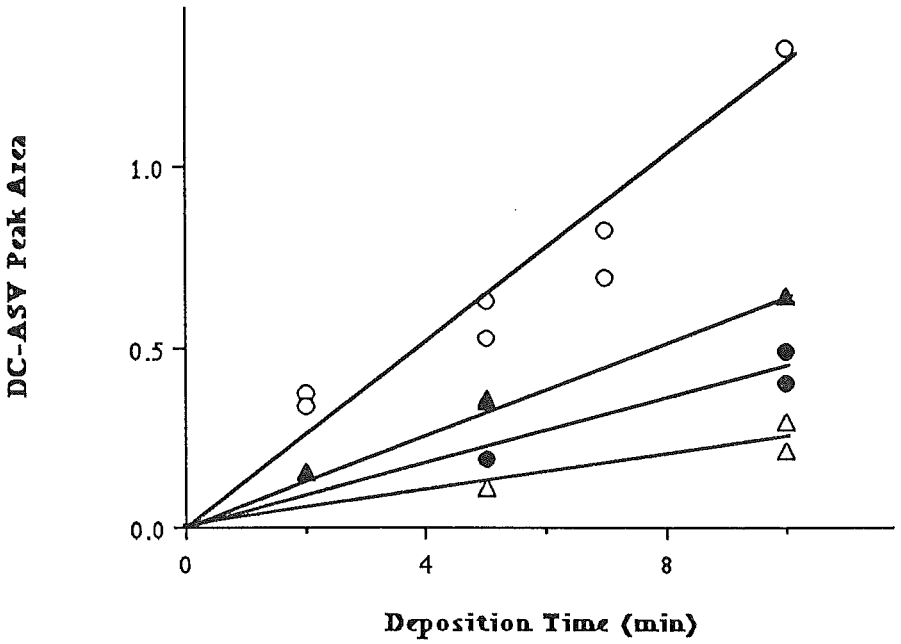


Figure 8.2: Effect of Humic Substances on the DC-ASV Lability of $Cu(PAN)_2$.

O, O ppm humic substance; ▲, 2 ppm SHHA; ●, 6 ppm SHHA; Δ, 30 ppm FA4

Table 8.15: Suppression of Cu(II) ASV Peak Area by Humic Substances

Humic Sample	Concentration	% decrease in peak area
SHHA	2 ppm	46
(0.025 μm filtered)[a]	6 ppm	64
	10 ppm	75
FA4	10 ppm	39
	30 ppm	78
	50 ppm	89

[a]similar results were obtained with unfiltered humic acid.

These data indicate that the decrease in peak area in the presence of humic acid was approximately two times greater than that in the presence of fulvic acid (on an equal concentration basis).

8.6.2 Association Capacities of Humic Substances for Cu-PAN and Cu-Oxine

A second series of experiments was performed in which increments of Cu(II) and ligand (L) were added (at a constant ratio) to the analysis cell either in the presence or absence of humic and fulvic acids. The ASV peak current was recorded for deposition from each solution.

With Cu-PAN, initial experiments used a 1:2 Cu:PAN ratio but data were not reproducible. This may have resulted from precipitation of the sparingly soluble complex as the concentration of metal and ligand increased, or from adsorption on the electrode surface. Hence, a 1:1 Cu(II):PAN ratio was used for subsequent experiments (4.9×10^{-7} to 2.1×10^{-6}

mol L^{-1} Cu(II); 4.9 x 10^{-7} to 2.2 x 10^{-6} mol L^{-1} PAN). A 1:2 Cu(II):ligand ratio was used for all Cu-oxine experiments and no precipitation problems were apparent with this system (1.5 x 10^{-7} to 2.3 x 10^{-6} mol L^{-1} Cu(II); 2.9 x 10^{-7} to 5.3 x 10^{-6} mol L^{-1} oxine). The same "association capacities" were obtained for humic substances when either a 1:2 or a 1:1 Cu(II):oxine ratio was used.

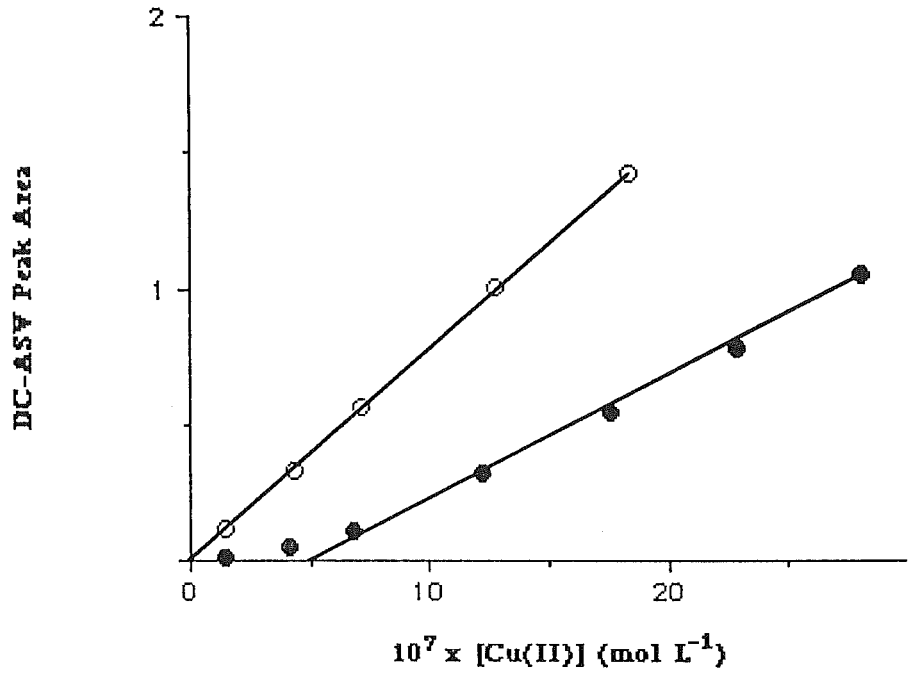


Figure 8.3: Association Capacity Curve for Addition of $Cu(oxine)_2$ to 0.1 M KNO_3, 0.01 M Tris (pH 8.2) in the Absence, O, and Presence, ●, of 50 ppm SHHA

In the presence of the copper complex (PAN or oxine), a plot of Cu(II) DC-ASV peak area *versus* [Cu(II)] gave a straight line passing through the origin; an inflexion was observed in the presence of humic substances. This is illustrated for Cu-oxine in Figure 8.3; similar curves were obtained for Cu-PAN. Extrapolation of the linear portion of the curve to the x-axis was taken as the "association capacity". The term "association capacity" is used because the ASV technique cannot differentiate between several possible processes which may be occurring, *viz:* sequestering of Cu(II) from Cu-L by humic molecules; sequestration of Cu-L by humic moieties; or formation of a ternary humate-Cu-L complex. All of these

processes could result in copper(II) species which are less labile and/or have a lower diffusion coefficient than does Cu-L.

Table 8.16: Association Capacities of Humic Substances for Cu-PAN and Cu-oxine Complexes (pH 8.2)

		Association Capacity[a]	
Humic Sample	Concentration	Cu-PAN	Cu-oxine
SHHA	6 ppm	4.8×10^{-7}	0
(0.025μm filtered)	50 ppm	nd	3.3×10^{-7}
SHHA	2 ppm	5.0×10^{-7}	nd
(unfiltered)	6 ppm	1.1×10^{-6}	nd
	6 ppm[b]	4.5×10^{-7}	nd
	10 ppm[b]	8.4×10^{-7}	nd
	10 ppm	nd	2.0×10^{-7}
	30 ppm	nd	3.1×10^{-7}
	50 ppm	nd	5.4×10^{-7}
	50 ppm[b]	nd	1.4×10^{-7}
FA4	5 ppm	2.8×10^{-7}	nd
	15 ppm	5.7×10^{-7}	nd
	50 ppm	nd	2.5×10^{-7}
	50 ppm[b]	nd	2.4×10^{-7}
	50 ppm[c]	nd	2.7×10^{-7}

[a] mol L^{-1}.
[b] 0.6 mol L^{-1} KNO_3.
[c] 1:1 Cu:oxine ratio.
nd = not determined.

The association capacities obtained by this technique are summarized in Table 8.16. Unless otherwise stated, the ionic strength was 0.1 mol L^{-1} KNO_3.

Larger capacities were observed with humic acid than with fulvic acid. Hence, at pH 8.2 humic acid was better at binding Cu-L, or at sequestering Cu(II) from Cu-L than was fulvic acid. Further, both humic and fulvic acid exhibited a greater association capacity for Cu-PAN than for Cu-oxine.

Filtered SHHA had *ca.* 0.5 times the capacity to interact with Cu-PAN than did whole SHHA. Whole SHHA had *ca.* 4 times the capacity of FA4 and, filtered SHHA had *ca.* 1.5 times the capacity of FA4. Similar ratios were obtained with Cu-oxine: filtered SHHA had *ca.* 0.6 times the capacity of unfiltered SHHA, whole SHHA had *ca.* 2.7 times the capacity of FA4, and filtered SHHA had *ca.* 1.6 times the capacity of FA4. An increase in ionic strength from 0.1 to 0.6 mol L^{-1} KNO_3 had no effect on the ability of fulvic acid to associate with Cu-oxine, but did affect humic acid association capacities. At 0.6 mol L^{-1} there was a 3.8 fold decrease in the association capacity of unfiltered humic acid for Cu-oxine, and a 2.4 fold decrease in that for Cu-PAN.

8.7 DISCUSSION

8.7.1 Effect of Humic Substances on the Apparent Lability of Cu-PAN

The DC-ASV peak area measured for a 1:2 Cu:PAN solution was substantially decreased in the presence of SHHA or FA4 (Figure 8.2, Table 8.15). It is noted that the percentage decrease quoted is relative to the 'apparent lability' of Cu-PAN in the absence of humic substances. (The apparent lability of Cu-PAN (1 x 10^{-7} mol L^{-1} Cu(II), 2 x 10^{-7} mol L^{-1} PAN) in 0.6 mol L^{-1} KNO_3 and 0.01 mol L^{-1} Tris buffer (pH 8.2) was 75%; Table 8.14).

The decrease in apparent lability of Cu-PAN in the presence of humic substances is consistent with the formation of a new Cu(II) complex or adduct which is less labile than $Cu(PAN)_2$ and/or has a lower diffusion coefficient. This observation does not differentiate between sequestration of Cu(II) by the humic substance, displacement of one ligand to form

a ternary complex, or hydrophobic adduct formation; neither does the result that humic acid caused a larger decrease in lability than did fulvic acid. The more hydrophobic humic acid has the higher average molecular weight; therefore, any new copper(II) species (a hydrophobic adduct or a binary or ternary humic complex) will have a smaller diffusion coefficient. Humic acid also forms stronger complexes with Cu(II) than does fulvic acid (Chapter 6).

8.7.2 Association Capacity of Humic Substances for Cu-PAN and Cu-oxine

The association capacities determined at a NCTMFE are consistent with algal assays. Specifically, they indicate that 5 ppm humic acid could sequester the amount of "Cu-oxine" which was present in the algal assays. It is noted that both techniques (i.e. ASV and algal assays) cannot distinguish hydrophilic complexation of Cu(II) from association of humic moieties with the intact hydrophobic metal complex. Although the concentrations of both metal and ligand employed in the ASV experiments were 10 times greater than those used in the assays (3.1×10^{-8} mol L^{-1} Cu(II), 6.2×10^{-8} mol L^{-1} ligand), a linear extrapolation from the NCTMFE association capacities can probably be assumed (however, the equilibrium position will be different in each case).

In all cases, for both Cu-oxine and Cu-PAN, the slope of the linear portion of the plot of Cu(II) DC-ASV peak area *versus* [Cu(II)] was reduced by approximately 45% in the presence of humic substances; Figure 8.3. This may indicate that the stronger binding which gives rise to the 'association capacity' is followed by utilization of a larger number of more weakly binding sites (or sites on molecules with lower molecular weight). This result is consistent with observations on the complexation capacity of river water for Cu(II) (Morrison & Florence, 1989a).

With Cu-oxine, extrapolation of the NCTMFE results indicates that 1 ppm unfiltered humic acid, and 5 ppm fulvic acid, can sequester 1×10^{-8} - 2×10^{-8} mol L^{-1} "Cu-oxine". This would not be sufficient to completely ameliorate toxicity in the algal assays. However, some impact was observed with algal cell numbers remaining constant for the duration of the assay, as opposed to cell death. 5 ppm humic acid (at 0.1 mol L^{-1} ionic strength) can

"complex" 5.4 x 10^{-8} mol L^{-1} "Cu-oxine", which is sufficient to tie up all the oxine-bound copper(II) present in algal assays.

With Cu-PAN, the NCTMFE association capacities indicated that both humic and fulvic acid, at 1 ppm, should be able sequester all the "Cu-PAN" present in algal assays. However, the toxicity of Cu-PAN was not inhibited under any conditions. Differences in the properties of the ligands oxine and PAN may explain this observation. In contrast to oxine, PAN itself is toxic to *Nitzschia closterium*. Therefore, if humic substances displace PAN from the Cu-PAN complex then a toxic effect will still be exerted by the free ligand. This assumes that humic molecules do not associate with free PAN.

8.7.3 Effect of Filtration of Humic Acid

The difference between 0.025 μm filtered humic acid and whole humic acid samples is interesting. As the samples were adjusted to equal concentrations in these experiments, these results suggest that the higher molecular weight/ colloidal humic acid moieties contain functional groups with higher affinities for Cu(II). In turn, components of filtered humic acid have a greater Cu(II) affinity than do fulvic acid moieties. Further, this indicates that the inability of filtered 5 ppm SHHA to ameliorate Cu-oxine toxicity may not be due only to a lower concentration of humic acid in the filtered sample. That is, the particulate/colloidal fraction of humic acid may contain components with a stronger affinity for Cu(II) than does the filtered fraction. This proposal is consistent with results from ISE potentiometric studies, which indicated stronger Cu(II) binding by an unfiltered humic acid sample (Chapter 6).

Consistent with this proposal, there is some evidence in the literature that higher molecular weight components of humic substances have a greater affinity for metal ions. For example, the conditional stability constants for complexation of Cu(II) by "smaller" fulvic acid molecules were reported to be only 2% of that for the "larger" molecules (Rainville & Weber, 1982). Sedlacek et al. (1989) observed that the lowest molecular weight moieties of an aquatic humic sample (< 10^3 Dalton) could not reduce cadmium(II) toxicity to algae (*Selenastrum capricornatum*) as effectively as did the higher molecular weight fraction. A similar result was reported by Giesy et al. (1977).

8.7.4 Effect of Ionic Strength on Association Capacities

At 0.6 mol L^{-1} (KNO_3), the ionic strength of seawater, the ASV measurements indicated that the association capacity of humic acid for both Cu-PAN and Cu-oxine was decreased. These results suggested that not enough "Cu-oxine" would be sequestered by humic acid to inhibit toxicity. This contrasts with the fact that complete amelioration of Cu-oxine toxicity by 5 ppm humic acid was observed for the algal assays conducted in seawater. It is possible that there are some natural chelators in seawater which complement the effect of added humic substances. For example, a study by Apte et al., (1990) reported that the organic ligands in seawater had a complexation capacity for Cu(II) in the range 2×10^{-8} - 7×10^{-8} mol L^{-1}; in estuarine samples the capacity was over 2×10^{-7} mol L^{-1}.

The decrease in association capacities at higher ionic strength is not consistent with a hydrophobic binding mechanism. The association of humic acid with hydrophobic substrates such as DDT *increases* with ionic strength. At higher ionic strength the humic polymer becomes less hydrophilic because charges are neutralized; hydrophobic compounds then preferentially associate with the uncharged moieties (Carter & Suffet, 1982).

In support of a hydrophilic mode of interaction between humic substances and the copper complexes, ISE potentiometric studies on the complexation of Cu(II) by humic substances indicated apparent weaker binding in 0.6 mol L^{-1} KNO_3 as compared to an 0.1 mol L^{-1} KNO_3 medium (Chapter 6).

8.7.5 Mechanism of Interaction of Humic Substances with Cu-PAN and Cu-oxine

The association capacities for humic acid were larger than those for fulvic acid. Although this is consistent with the greater hydrophobicity of humic acids, one might predict insignificant hydrophobic interaction with fulvic acids at pH 8.2 (Kile & Chiou, 1989a). However, this result contrasts with that from ISE potentiometric studies (Chapter 6) which have established that at the same concentration (ppm) SHHA and FA4 have equal copper(II) complexation capacities (pH 7.0) and form binary copper(II) complexes of similar

stability (pH 2.5 - 7.5). Further, for both SHHA and FA4 the association capacity was greater for Cu-PAN than for Cu-oxine. This is not consistent with hydrophobic binding.

Both these observations are consistent with a hydrophilic mode of interaction between humic substances and the copper(II) complexes, involving either sequestration of Cu(II) or displacement of a low molecular weight ligand to form a ternary complex. Further, the different capacities of humic substances for each of these complexes indicates that there is a *range* of functional groups in humic substances which can complex Cu(II) strongly.

These observations have important environmental implications. If the action of humic substances on hydrophobic metal complexes is the hydrophilic complexation of the metal ion then this interaction will have an impact on the toxicity of these metal species only if the displaced ligand is not toxic. Furthermore, the less stable is a particular metal complex, the more readily its toxicity will be inhibited by humic substances.

EFFECT OF HUMIC SUBSTANCES ON METAL COMPLEX ABSORPTION SPECTRA

8.8 INTRODUCTION

Results from algal assays established that humic acid can ameliorate the toxicity of Cu-oxine to *Nitzschia closterium*. DC-ASV studies established that humic and fulvic acids interact with Cu-PAN and Cu-oxine. These results were consistent with a hydrophilic mode of interaction between the humic substance and the copper(II) complex.

Spectrophotometric measurements were performed to further elucidate the mechanism of interaction of humic substances with Cu-PAN. Changes in the visible spectrum of this complex in the presence of humic substances should be able to easily distinguish between displacement of one PAN molecule (to form a ternary HS-Cu-L complex) or sequestration of Cu(II) by the humic substances, releasing two PAN molecules.

8.9 RESULTS

As reported in Section 8.3.1, the limiting stoichiometry of the Cu-PAN reaction at pH 8.2 was established to be 1:2. Spectrophotometric parameters for this reaction were: CuL, λ_{max} 550 nm, ε_{max} 13 500; CuL_2, λ_{max} 550 nm, ε_{max} 22 500.

Addition of increments of humic substances (0 - 50 ppm) to a 1:2 Cu-PAN complex resulted in a decrease in the absorption at 550 nm, and an increase in that at 475 nm. Spectra were measured 'by difference' (0 - 50 ppm humic substance in test and reference cells) in 50 mm cells. Representative spectra are shown in Figure 8.4 for humic acid; similar spectra were obtained for fulvic acid.

The limiting changes in the spectra were achieved for 20 ppm FA4 and 10 ppm SHHA (for $[Cu(PAN)_2] = 3 \times 10^{-7}$ mol L^{-1}).

Addition of humic substances to a 1:1 Cu-PAN complex (3×10^{-7} mol L^{-1}) resulted in a decrease in the absorption at 550 nm (25% for a 10 or 20 ppm FA4 solution), but no increase in that at 475 nm.

Measurements in 0.6 mol L^{-1} $KNO_3/10^{-3}$ mol L^{-1} Tris buffer were attempted but very poor spectra were obtained; this is indicative of precipitation of the Cu-PAN complex and/or of the humic substance at high ionic strength.

8.10 DISCUSSION

These spectrophotometric studies on the interaction of humic substances with Cu-PAN established that formation of a ternary HS-Cu-L complex occurs, consistent with the ASV results.

The spectra obtained were not of very high quality because of the significant correction for the strong absorption by humic substances (this precluded study of the Cu-oxine system in the UV), the necessity to use low concentrations of metal complex and thus long path length cells and a very sensitive absorbance scale on the spectrophotometer,

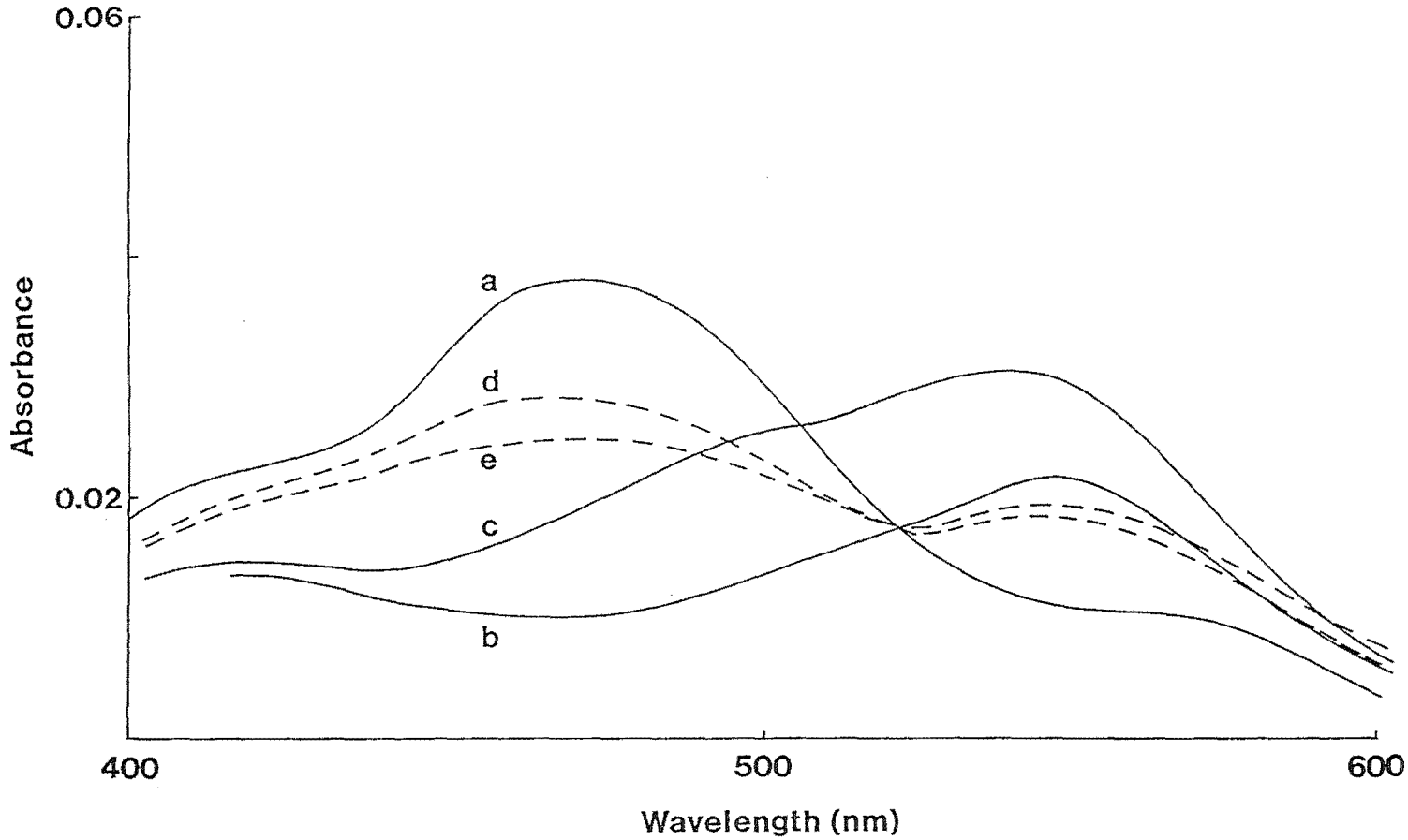


Figure 8.4: Visible Absorption Spectra for Cu(II)-PAN Complexes in 0.01 M Tris, pH 8.2

PAN (6×10^{-7} M), (a); CuPAN (3×10^{-7} M), (b);
$Cu(PAN)_2$ (3×10^{-7} M) in the absence (c), and presence of
Humic Acid: 10 ppm (d), 40 ppm (e).

and the need to 0.025 μm membrane filter the humic acid solutions, with the associated possibility of contamination.

The absorption at 550 nm is decreased in the presence of humic substances but not completely eliminated (Figure 8.4). This is consistent with conversion of CuL_2 to a CuL moiety (ε_{max} CuL ≈ 0.6 x ε_{max} CuL_2). This is confirmed by the increased absorption at 475 nm which indicates partial release of PAN from the complex. The limiting changes in the spectra in the presence of humic substances are consistent with formation of a single product of fixed stoichiometry.

Further supporting evidence for the formation of a ternary HS-Cu-PAN complex was provided by the effect of humic substances on a 1:1 Cu-PAN solution (3 x 10^{-7} mol L^{-1}). In the presence of humic substances, the absorbance at 550 nm was decreased indicating some interaction, but from the absorbance at 475 nm there was no evidence for release of PAN from the complex.

The Cu-PAN spectra were not of high quality, but much better data were obtained by use of the 1:1 and 1:2 Cu-PAR complexes (3 x 10^{-7} mol L^{-1} CuL_2; λ_{max} 518 nm, ε_{max} 38 600, λ_{isos} 458 nm). (PAR (4-(2-pyridylazo)-resorcinol) is a more water soluble derivative of PAN). The effect of humic substances on the spectra of these Cu-PAR complexes confirmed the Cu-PAN results, *viz:* humic substances displace a low molecular weight ligand to form a ternary complex (spectra not shown). Experimental details were the same as those for Cu-PAN. Although disparate values of log K_1 have been reported for Cu-PAR (*viz:* 11.7 (Iwamoto, 1961); 17.22 (Funahashi et al., 1971)), it is expected that the Cu-PAR complexes will have similar stability to those of PAN. For example, log K_1 for Zn(II) and Mn(II) PAR complexes was 1.2 log units greater than that for the corresponding PAN complexes (Corsini et al., 1962). Limiting changes in the Cu-PAR spectra occurred at the same humic substance concentrations as reported for Cu-PAN.

It is of interest to compare the effect of humic substances on the 1:2 Cu-PAN complex with that for ligands which have been proposed as models for the chelating moieties in humic substances. Thus, for 1:2 Cu-PAN *and* Cu-oxine (3 x 10^{-7} mol L^{-1} Cu(II), 6 x 10^{-7} mol L^{-1} ligand), the presence of up to 5 x 10^{-4} mol L^{-1} citric acid, or 1.8 x 10^{-4} mol L^{-1} salicylic acid

had no effect on the absorption spectrum. These observations indicate that there are some functional groups in humic substances which bind copper(II) more strongly than do these "model" ligands. This aspect is discussed further in Chapter 6.

The concentration of Cu-PAN used in these spectrophotometric measurements is within the range of values used in the ASV association capacity measurements (Figure 8.3), and is ten times greater than that used for the algal assays. The observed spectrophotometric changes are consistent with the association capacities measured by DC-ASV (i.e. the association capacity for 15 ppm FA4 was 5.7×10^{-7} mol L^{-1} Cu-PAN; that for 6 ppm SHHA (0.025 μm filtered) was 4.8×10^{-7} mol L^{-1}; Table 8.16). Further, these results indicate that the effect of humic substances on a 1:1 Cu-PAN complex (as used in the association capacity measurements) is similar to that on a 1:2 Cu-PAN complex (spectrophotometric studies).

8.11 ENVIRONMENTAL SIGNIFICANCE

It is of interest to extrapolate these experimental data to the environmental situation. The question that arises is, can humic substances ameliorate the toxicity of hydrophobic metal complexes in soils and natural waters? Several parameters must be considered in such an evaluation; these are discussed below.

8.11.1 Ionic Strength

The algal assays were conducted in seawater medium (ionic strength, 0.6 mol L^{-1}). Under these conditions humic acid has limited solubility and may also have a reduced capacity for binding transition metal ions because of competition from calcium(II). In contrast, partitioning-like interactions between humic acid and hydrophobic organic compounds have been reported to increase with increasing ionic strength (due to neutralization of charge on the humic polymers and/or salting out of the nonpolar compound) (Carter & Suffet, 1982; Thurman, 1985). Increased hydrophobic bonding between humic

molecules at higher ionic strength may create nonpolar regions into which lipid-soluble compounds can partition.

Dependence of Molecular Size on Ionic Strength

The solubility of humic and fulvic acids, and that of the hydrophobic metal complex will be affected by ionic strength. Fulvic acid is less affected by increased salinity than is humic acid; Alberts et al. (1989) observed that humic and fulvic acids have different salting-out characteristics on mixing of ocean water with freshwater containing estuarine humic matter. The larger humic acids were more susceptible to aggregation at higher salt concentrations. Fox (1983) observed that in an estuarine environment, 30 to 60% of the high molecular weight organic substances was removed by salting out. Conformational changes which occur when humic substances enter estuarine waters cause a reduction in size; these changes are more important for the higher molecular weight fractions. The percentage of soluble material in the high molecular weight fraction decreased with increasing salinity; contraction or coiling of humic acid occurred at high salinity. Most of the humic acid fraction may aggregate at intermediate salinities; in contrast, fulvic acid is largely unaffected (Mayer, 1985). In support of this, equilibrium ultracentrifugation measurements indicated that the apparent molecular weight of a surface water fulvic acid sample is independent of salt concentration (0.1 to 1.0 mol L^{-1} NaCl); whereas the molecular weight of peat and surface water humic acid samples increases significantly over the same ionic strength range (Reid et al., 1990). According to Reid et al. (1990), such an increase in molecular weight with ionic strength cannot be attributed predominantly to aggregation.

Ogura (1974) used ultrafiltration to study the molecular "weight" fractionation of dissolved organic matter (DOM) in coastal seawater. DOM with a molecular "weight" less than 500 (units not stated; defined as material which passed through a Diaflo UM-05 membrane) accounted for 24 - 42% of the total DOM; 8 - 23% of the total DOM had a molecular "weight" greater than 100 000 (material retained on a Diaflo XM-100 membrane).

Effect of Competing Ions on Metal Complexation by Humic Substances

Seawater contains a high concentration of calcium and magnesium ions (0.012 and 0.0532 mol L^{-1} respectively; Holland, 1978) which may compete, albeit weakly, for copper binding sites on the humic molecules (Winner & Gauss, 1986). The effect of water hardness and humic acid on the toxicity of copper to Daphnids was evaluated by Winner (1985). The ameliorating effect of humic acid on copper toxicity was similar in soft and medium water, but less in hard water. It was proposed that in hard water Ca(II) and Mg(II) displace Cu(II) from humic acid coordination sites.

In river waters, approximately 20% of humic metal binding sites are unassociated with cations; the remainder are occupied primarily by calcium, and to a lesser extent, by magnesium (Mantoura et al., 1978). In seawater, a major fraction of humic carboxylate sites are bound to alkaline earth cations. It is unclear whether or not humic substances become completely saturated with cations on mixing with seawater. Calculations based on published stability constants give conflicting results with one set of data predicting complete saturation (Mantoura et al., 1978), while another predicts that about one third of the acidic sites will be dissociated (Mantoura & Woodward, 1983). Further, stability constant data suggest that the complexation of trace metals by humic substances should decrease with increasing salinity, resulting in a negligible influence of humic matter on trace metal speciation at high salinities (Mayer, 1985). Schnitzer and Hanson (1970) reported a marked effect of ionic strength on the stability constants for fulvic acid complexes with a range of metal ions. A linear decrease in the stability constant was observed as the ionic strength was increased from 0 to 0.1 mol L^{-1}. However, field data have suggested that relatively high concentrations of copper are complexed by organic ligands at intermediate or high salinities in estuarine zones. Coastal surface seawater typically has 40 to 60% of total copper present as inert organic complexes (Florence, 1982, 1986). In estuarine zones, organic copper complexes may represent 14 to 70% of the total dissolved copper (Mills & Quinn, 1984). These results again highlight the need for caution when calculations based on stability constants are used to predict metal speciation in the environment.

The effect of divalent cations on copper complexation may be electrostatic and/or the result of direct competition for binding sites (Cabaniss & Shuman, 1988a). The effect of Mg(II) on the lability of SHHA-Cu(II) complexes at pH 4.8 was investigated in this work. At a humic acid concentration of 1 mg L^{-1}, and 1.2 x 10^{-7} mol L^{-1} Cu(II), approximately 40% of copper(II) was labile at a hanging mercury drop electrode at pH 4.8 (see Chapter 7). Addition of Mg(II) up to 1.92 x 10^{-5} mol L^{-1} (160 x [Cu(II)]) had no effect on the apparent Cu(II) lability. At 1.94 x 10^{-3} mol L^{-1} Mg(II) there was a 6% increase in labile Cu(II), and a 14% increase in the presence of 1.16 x 10^{-2} mol L^{-1} Mg(II) (*ca.* 10^5 x [Cu(II)]). In seawater the concentration of magnesium is *ca.* 1.7 x 10^6 times greater than the typical concentration of copper; these results indicate that complexing by Mg(II) may affect the ability of humic substances to complex with copper. Calculations performed with the equilibrium program SIAS, using citric acid as a model for humic chelating groups, suggested that the percentage Cu(II)-citrate complex formation would decrease in favour of Mg(II)-citrate as the concentration of magnesium increases. However, although ISE potentiometric studies indicated that the 'apparent' Cu(II) binding strength of humic substances was decreased in the presence of Mg(II), strong Cu(II) complexation at pH 8.2 may not be altered significantly (Chapter 6).

Calcium and magnesium have a measurable effect on copper binding by Suwannee River fulvic acid. Decreases in pCu (i.e. increases in free Cu(II)) of up to 0.3 units at 1 x 10^{-3} mol L^{-1} Ca(II) or Mg(II), and up to 0.6 pCu units at 1 x 10^{-2} mol L^{-1} Ca(II) or Mg(II) were observed at pH 8 (Cabaniss & Shuman, 1988a). Increasing the ionic strength from 0.01 to 0.10 mol L^{-1} ($NaClO_4$) decreased pCu by 0.7 to 1.0 log units at pH 8.44 (Cabaniss & Shuman, 1988a).

This observation was confirmed in the present work. ISE potentiometric studies on the complexation of Cu(II) by humic substances indicated that the 'apparent' Cu(II) binding strength was decreased in 0.6 mol L^{-1} KNO_3 media (Chapter 6). This decrease in copper complexing by fulvic acid with increased ionic strength is interesting and is important for speciation predictions in natural systems. Extrapolation of ASV and ISE results (which utilize high background electrolyte concentrations) to low ionic strength freshwater

conditions may greatly overestimate the expected activity of copper in the environment. In a related study, variations in the source of organic matter were found to be less important than chemical factors such as pH, ionic strength and cation binding in predicting pCu in natural waters (Cabaniss & Shuman, 1988b).

Conclusions

It is generally anticipated that the majority of copper(II) ions will be complexed by humic substances at lower ionic strength. Assuming that humic substances ameliorate the toxicity of hydrophobic copper complexes by sequestering the Cu(II) ion, then they should have a greater impact on the toxicity of these species in estuarine zones (where the concentration of humic material is also higher) than in open-ocean seawater. In turn, an even greater ameliorating effect may be expected in freshwater systems where the concentrations of metal cations are low and humic substances are typically present in higher concentrations (4 to 20 ppm in rivers and lakes, 30 to 60 ppm in marshes and bogs; Thurman, 1985). However, at the lower pH of freshwaters, as compared to seawater, humic substances may have a reduced affinity for copper(II) (Mills et al., 1982).

The toxicity of lipid-soluble copper complexes to the freshwater algae *Chlorella pyrenoidosa* has been demonstrated (Stauber & Florence, 1987, 1989); it would be of interest to investigate the impact of humic substances on this toxicity. Further, it would be of environmental significance to extend these studies to organisms other than algae; for example, marine amphipods (Ahsanullah & Florence, 1984).

8.11.2 Source and Purity of Humic Substances

This work has shown that humic acid, by virtue of its strong affinity for copper(II) ions, can dissociate and detoxifiy hydrophobic copper complexes (provided that the displaced ligand itself is not toxic). However, the soil-derived humic substances used in this study are relatively "pure". That is, they have a low ash content and are reasonably free from metallic impurities. In contrast, the strong binding sites of humic substances in soils and natural waters may already be saturated with metal ions and hence less able to sequester an influx

of contaminant. Furthermore, the soil humic substances used in this work are unlikely to be representative of marine humic materials (Malcolm, 1990).

Many uncertainties are involved in attempts to extrapolate experimental data to the environmental situation. In this work, humic acid was observed to have a greater ability to ameliorate the toxicity of hydrophobic copper complexes than did fulvic acid. Fulvic acid is the predominant fraction of dissolved humic substances in natural waters, whereas humic acid is most likely to be associated with colloidal/particulate phases.

8.11.3 Applicability to Other Metal Complexes

Of the predominant divalent metal ions in soils and natural waters, copper is the only one which is likely to form stable organic complexes (either polar or nonpolar) at environmentally significant concentrations of metal and ligand. Lead has an affinity for inorganic adsorbents such as colloidal ferric hydroxide and silica (Florence, 1983), cadmium forms very stable chloro-complexes, and zinc exists predominantly as a dihydroxy complex (Florence & Batley, 1976).

Spectrophotometric measurements indicated that cadmium and lead, at 10^{-7} mol L^{-1}, do not complex even with sulphur-donor ligands such as DDTC, APDC, ethylxanthogenate and TAN. In addition, humic substances have a much higher affinity for Cu(II) than for Zn(II), Cd(II) or Pb(II). That is, complex formation by these other environmentally significant trace metal ions with hydrophobic ligands is of insufficient stability to allow their existence at environmental concentrations.

The toxicity of a metal complex is determined by its stability and lipid solubility (Florence et al., 1984). In this work, the complexes Cu-PAN and Cu-oxine were chosen because they are stable, lipid-soluble and extremely toxic to algae. Even though calculations based on published stability constants indicated that these complexes are very stable, humic substances ameliorated their toxicity by hydrophilic complexation of Cu(II), resulting in displacement of a low molecular weight ligand and formation of a ternary complex. At typical environmental concentrations of ligands and metals most copper(II) complexes with simple

ligands will be significantly less stable than Cu(II)-oxine complexes and will have the 1:1 stoichiometry of Cu-PAN.

Humic substances can ameliorate the toxicity of lipid-soluble copper complexes only if the ligand itself is not toxic. The less stable the copper complex, the more readily its toxicity should be ameliorated by humic substances. The results from this study should be applicable to other toxic copper complexes in the environment. Several ligands which form lipid-soluble complexes with copper are common pollutants in soils and natural waters. For example, copper-triethanolamine and oxine are fungicides, ethylxanthogenate is a mineral flotation agent, and 2,9-dimethyl-1,10-phenanthroline is typical of compounds present in oil shale and coal liquefaction process waters (Ahsanullah & Florence, 1984).

SECTION B: EQUILIBRIUM DIALYSIS

8.12 INTRODUCTION

Equilibrium dialysis has been used to study the association of humic substances with nonpolar pollutants (Carter & Suffet, 1982; McCarthy & Jimenez, 1985a). In the present work, dialysis was used to probe the interaction of humic and fulvic acids with the hydrophobic metal complexes Cu-PAN and Cu-oxine. However, this technique was found to have inadequate sensitivity for these measurements. The details of these experiments are now briefly discussed.

8.13 EXPERIMENTAL

All experiments were performed in a class 100 clean room, micropipettes with disposable plastic tips were used to dispense solutions, and disposable gloves were worn during manipulation of the dialysis tubing. It was established that the micropipette tips did not leach any measurable copper(II). Tris buffer was purified electrolytically by electrolysis

at a mercury pool cathode at -1.0 V. Spectrapor 6, 1 000 MWCO tubing and Spectrum dialysis closures were prepared as described previously (Chapter 5).

Each dialysis experiment was performed in an acid-washed perspex cell covered with Parafilm. 2.0 mL of humic substance solution (in 0.005 mol L^{-1} Tris, pH 8.2) inside dialysis tubing was dialyzed against 20.0 mL of Cu-oxine or Cu-PAN solution (3 x 10^{-8} mol L^{-1} Cu(II), 3 x 10^{-6} mol L^{-1} ligand, in 0.005 mol L^{-1} Tris, pH 8.2). The solutions were allowed to equilibrate for 24 h (stirred continuously with a Teflon-coated magnetic stirrer bar) then the concentration of Cu(II) in the dialyzate and in the retentate was measured *via* DC-ASV at a NCTMFE in acid solution (20 µL Aristar HNO_3 per 5 mL of solution).

In all experiments the humic acid solution was not filtered before use.

8.14 RESULTS

8.14.1 Dialysis of Humic Substances with Cu-PAN

Table 8.17: Dialysis Studies on the Interaction of Humic Substances with Cu-PAN, pH 8.2

Solution inside tubing	[Cu(II)] outside tubing[a]	[Cu(II)] inside tubing[a]
5 ppm SHHA	5.3 x 10^{-7}[b]	5.4 x 10^{-7}
	4.9 x 10^{-7}	1.1 x 10^{-6}
25 ppm SHHA	5.6 x 10^{-7}	1.1 x 10^{-6}
	2.2 x 10^{-7}	4.8 x 10^{-7}
	3.9 x 10^{-7}	5.3 x 10^{-7}
25 ppm FA4	1.7 x 10^{-7}	9.0 x 10^{-7}
	3.5 x 10^{-7}	1.0 x 10^{-6}

[a] mol L^{-1}.

[b] each row represents a separate experiment.

The concentrations of Cu(II) found inside and outside the dialysis tubing when humic and fulvic acid were equilibrated with Cu-PAN are given in Table 8.17.

8.14.2 Dialysis of Humic Substances with Cu-oxine

The concentrations of Cu(II) found inside and outside the dialysis tubing when humic and fulvic acid were equilibrated with Cu-oxine are given in Table 8.18.

Table 18: Dialysis Studies on the Interaction of Humic Substances with Cu-oxine, pH 8.2

Solution inside tubing	[Cu(II)] outside tubing[a]	[Cu(II)] inside tubing[a]
5 ppm SHHA	5.3×10^{-7}[b]	5.4×10^{-7}
	6.1×10^{-7}	8.5×10^{-7}
25 ppm SHHA	6.1×10^{-7}	8.8×10^{-7}
	3.5×10^{-7}	4.6×10^{-7}
5 ppm FA4	6.7×10^{-7}	1.4×10^{-6}
	1.5×10^{-7}	2.9×10^{-7}
25 ppm FA4	3.2×10^{-7}	8.9×10^{-7}

[a] mol L^{-1}.

[b] each row represents a separate experiment.

8.14.3 Sources of Copper(II) contamination

The concentrations of copper(II) measured in the dialysis solutions were much higher than the concentration of Cu(II) originally added (by up to a factor of 45). To trace the source of copper(II) contamination, individual components of the system were placed in the perspex cells, in a 0.01 mol L^{-1} Tris solution (pH 8.2), and stirred for 24 h. The concentration of Cu(II) in the resultant solution was then measured (as described above).

The copper content of a solution in the perspex cell with (i) a magnetic stirrer was 5.9×10^{-8} mol L^{-1}, (ii) dialysis closures and a magnetic stirrer was 2.6×10^{-8} mol L^{-1}, and (iii) dialysis tubing and a magnetic stirrer was 9.3×10^{-8} mol L^{-1}.

Some dialysis experiments were also performed with humic substances and oxine, with no added copper(II). Results are given in Table 8.18.

Table 8.18: Dialysis Studies on the Interaction of Humic Substances With Oxine: No Added Copper(II) (pH 8.2)

Solution inside tubing[a]	[Cu(II)] outside tubing[b]	[Cu(II)] inside tubing[b]
25 ppm SHHA	5.4×10^{-7}	9.7×10^{-7}
0.005 M Tris	1.5×10^{-7}	3.6×10^{-7}
25 ppm FA4	1.8×10^{-7}	8.3×10^{-7}

[a]the solution inside the tubing in all experiments was 3×10^{-6} mol L^{-1} in oxine.

[b]mol L^{-1}.

8.15 DISCUSSION

The ratio of copper(II) inside and outside the dialysis tubing in the humic substance-Cu-oxine experiments was virtually the same as that obtained for oxine with no added copper (or humic substance); Table 8.18.

The dialysis tubing appears to be the main source of copper(II) contamination, even though it was cleaned exhaustively. The presence of ligands with an affinity for Cu(II) (e.g. humic substances,oxine and Tris) facilitated leaching of copper(II) from the tubing. Apte et al. (1989) reported a similar Cu(II) contamination problem associated with dialysis tubing.

The NCTMFE "titrations" discussed above (Table 8.16) indicated that 6 ppm unfiltered SHHA could complex 1.1×10^{-6} mol L^{-1} "Cu-PAN", and 50 ppm unfiltered SHHA could complex 5.4×10^{-7} mol L^{-1} "Cu-oxine". However, the dialysis results (Table 8.17) indicated no difference between the extent of association of humic acid with Cu-PAN and that with Cu-oxine. Further, an increase in the concentration of humic substance inside the dialysis tubing did not alter the distribution of Cu(II) across the membrane.

It is possible that the high concentration of ligand used in these experiments (to ensure complete Cu-L formation and minimize complexation of Cu(II) by the hydrophilic functional groups of the humic substance) "blocked" some of the sites in humic substances which may be involved in Cu(II) complexation. It is probably more likely that the amount of "Cu-L" associated with humic substances is too small to be reliably detected by this dialysis method due to the high background levels of copper(II) and the high affinity of the dialysis tubing for Cu(II).

CHAPTER 9

INTERACTION OF HUMIC SUBSTANCES WITH HYDROPHOBIC METAL COMPLEXES

9.1 INTRODUCTION

This chapter briefly describes several other techniques used to study the interaction of humic and fulvic acids with a range of hydrophobic metal complexes. In Chapter 8 it was established that humic substances interact with, and can detoxify hydrophobic Cu(II) complexes (by displacement of the low molecular weight ligand to form a ternary complex). Attempts were made to extend these results to complexes with metal ions other than Cu(II).

Specifically, the techniques used were: solvent extraction (in which an aqueous phase containing humic substances and a hydrophobic metal complex was equilibrated with chloroform), cathodic stripping voltammetry (CSV) (in which the current for reduction of a hydrophobic metal complex was measured in the absence and presence of humic substances), and equilibrium dialysis (in which humic substances inside dialysis tubing were dialyzed against a solution of a hydrophobic metal complex).

One aim of this work was to probe the possible existence of hydrophobic zones in humic acid aggregates and their ability to sequester hydrophobic metal complexes. Ideally, the metal complexes to be used in such studies should form appreciably at environmentally significant concentrations of metal and ligand, and be stable enough to prevent the hydrophilic functional groups in humic substances from competing for the metal ion. Humic substances form very stable complexes with Cu(II); they were able to interact hydrophilically with stable hydrophobic Cu(II) complexes (Chapter 8). Pb(II), Cd(II), and Zn(II) complexes with humic substances are considerably less stable than those of Cu(II); therefore, use of hydrophobic complexes of these metal ions should minimize the chance of competitive hydrophilic interaction.

Initially a wide range of complexes were considered; however, UV-visible spectroscopy established that several of these lacked the necessary stability to form at environmental concentrations. The metal complexes studied (which did have adequate

stability) were Cd(II) and Zn(II) complexes with 1-(2-pyridylazo)-2-napthol (PAN), and $[Co(DMG)_2NH_3Cl]$.

It was established that the techniques described above provided inadequate sensitivity and/or humic substances themselves interfered in the measurements preventing meaningful interpretation.

The reader is asked to refer to the frontispiece of this thesis before proceeding.

9.2 EXPERIMENTAL

9.2.1 Ligand Solutions

Humic substances are described in Chapter 3.

The PAN sample and solution preparation, and Tris buffer are described in Chapter 8. APDC (BDH) was used without further purification.

9.2.2 Metal Solutions

Cd(II) and Zn(II) solutions were prepared by dissolution of the appropriate weights of Analar salts in Milli-Q water (pH *ca.* 4, to prevent hydrolysis) ($ZnSO_4.7H_2O$, Prolab; $CdSO_4.8H_2O$, BDH).

9.2.3 CSV Measurements with $[Co(DMG)_2NH_3Cl]$

$[Co(DMG)_2NH_3Cl]$ was synthesized according to the method of Jolly (1968); a standard solution was prepared by dispersion in dilute alkali.

The PAR 303 HMDE and associated apparatus are described in Chapter 7. All experiments were performed in a class 100 clean room. The instrumental parameters were: stirring speed, 'fast'; accumulation potential, -0.70 V; and accumulation time, 2 min. For DC-ASV the scan rate was 20 mV s^{-1}; for DP it was 5 mV s^{-1}. The amount of $[Co(DMG)_2NH_3Cl]$ accumulated during the quiescent time (15 s) and the time for the cathodic scan was subtracted from all measurements. Following accumulation, the potential was scanned cathodically; the peak corresponding to reduction of the cobalt complex occurred at *ca.* -0.93 to -0.95 V (the peak position was shifted to more positive potentials in the presence of humic substances).

9.2.4 Solvent Extraction

All experiments were performed in a class 100 clean room. The metal-PAN complexes were formed in aqueous solution (5 mL 0.01 mol L^{-1} borax buffer, pH 9.18), in the presence or absence of humic substance, followed by extraction into 5 mL Spectroscopic chloroform (Riedel-de Haën). 25 mL acid-washed glass separating funnels were used. The chloroform was equilibrated with Milli-Q water before use. The concentration of metal complex extracted into the organic phase was quantified by visible absorption spectroscopy.

Humic substance solutions were not filtered before use. The humic solutions, and the borax buffer, were initially equilibrated with PAN/$CHCl_3$ to remove any metal impurities; the PAN was then removed by extraction with Spectroscopic chloroform.

9.2.5 Equilibrium Dialysis Utilizing Radiochemical Detection

These experiments were performed at the CSIRO Lucas Heights Research Laboratories, Sydney, in collaboration with Dr John J. Fardy, Ms Jenny L. Stauber, and Dr T. Mark Florence.

Measurement of Cd^{109} radioactivity was performed on 5 mL samples in acid washed polypropylene scintillation vials. A low resolution NaI gamma counter was used (this has the best efficiency for determining total counts at a known energy); the concentration of Cd^{109}, and the time period over which activity was counted, were chosen to give 10 000 to 20 000 counts. The background radiation (counts for distilled water) and a standard Cd^{109} solution were counted prior to measurement of the test solutions. The stability of the detector was monitored daily using a chi-squared test:

$$\chi^2 = \frac{\Sigma X^2 - \frac{\Sigma(x)^2}{10}}{\tilde{n}}$$

, where: ΣX^2 is the sum of squares for 10 readings; $\Sigma(x)^2$ is the sum of 10 readings, squared; and $\tilde{n}$ is the mean value for 10 readings. The detector was considered stable if the value of χ^2 was between 3.35 and 16.92.

The dialysis apparatus was described in Chapter 5; Spectrapor 6, 1 000 MWCO tubing was used. All experiments were performed in 0.1 mol L^{-1} Tris (pH 8.2). For each dialysis experiment, 2.0 mL of humic substance solution (in Tris buffer) inside dialysis tubing was dialyzed against 20.0 mL of $Cd^{109}(PAN)_2$ solution (3.40×10^{-10} mol L^{-1} $Cd(II)^{109}$, 3.15×10^{-7} mol L^{-1} PAN). A high concentration of PAN was used to ensure adequate complex formation. The radioactivity was monitored over time (5 mL of the dialyzate was removed for counting, and was subsequently returned to the dialysis cell) until stable values were obtained (20 - 30 h). At the end of the experiment the radioactivity in the dialyzate and the final retentate was counted.

There was no detectable radioactivity, above background levels, associated with the humic substance solutions.

9.3 RESULTS AND DISCUSSION

9.3.1 Stability of Metal Complexes at Environmental Concentrations

For many of the complexes considered to be suitable for these studies, calculations based on published stability constants (Perrin, 1979) indicated that significant complex formation would occur at environmental concentrations. (Ligands containing nitrogen or sulphur donor groups were chosen to maximize complex stability.) However, subsequent spectroscopic measurements established that this was not the case for most of the complexes studied, *viz:* Pb-oxinate; Cd(II) complexes of oxine, ammonium tetramethylenedithiocarbamate, diethyldithiocarbamic acid, 1-(2-thiazolylazo)-2-naphthol, 1,5-diphenyl-1,2,4,5-tetraazapent-1-en-3-thione (dithizone), and ethylxanthogenate; and bismuth(III) complexes of dithizone and N-nitrosophenylhydroxylamine.

This is an excellent illustration of the problems associated with use of stability constants in calculations for conditions outside the range in which the constants were determined.

9.3.2 CSV Measurements with $[Co(DMG)_2NH_3Cl]$

CSV was used to study the interaction between humic and fulvic acids and $[Co(DMG)_2NH_3Cl]$. This cobalt complex was chosen because it is nonionic, nonlabile, and can be studied at ppb levels by adsorption stripping voltammetry (Gilbert et al., 1988).

Two types of experiment were performed. Firstly, humic substances were added to a solution of the cobalt complex (8×10^{-8} mol L^{-1}), resulting in a significant decrease in the CSV stripping peak. For SHHA, the suppression was *ca.* 5% for a 2.0×10^{-3} mg mL^{-1} solution and *ca.* 67% for 1.9×10^{-2} mg mL^{-1}, using either DC or DP mode. For FA4, the suppression was *ca.* 5% for a 5.0×10^{-3} mg mL^{-1} solution, and *ca.* 35% for 1.9×10^{-2} mg mL^{-1}.

Secondly, increments of $[Co(DMG)_2NH_3Cl]$ were added to a solution of SHHA or FA4 (2.4×10^{-3} mg mL^{-1}) to generate concentrations in the range 3.5×10^{-8} to 6.5×10^{-7} mol L^{-1}. Experiments were performed with both DC and DP mode. There was no evidence of an inflexion in the plot of CSV stripping peak *versus* $[Co(DMG)_2NH_3Cl]$ concentration which would have been indicative of a 'complexation capacity' being attained (analagous to the complexation capacity for Cu(II); Chapter 6). A low concentration of humic substance was chosen in an attempt to minimize competitive adsorption on the mercury drop. However, at such low concentrations of humic substance it is possible that the 'complexation capacity' was exceeded after addition of the first increment of this cobalt complex. Therefore, to maximize the possibility of observing a complexation capacity, experiments were also performed with a higher concentration of humic acid (2.0×10^{-2} mg mL^{-1}) and with concentrations of $[Co(DMG)_2NH_3Cl]$ in the range 0.5×10^{-8} to 3×10^{-8} mol L^{-1}. Under these conditions the sensitivity was much lower, the baseline was very difficult to determine, and the high concentration of humic acid prevented use of DP mode. Nevertheless, the plot of CSV stripping peak *versus* concentration of cobalt complex again showed no evidence of an inflexion.

The adsorption of humic substances on the HMDE confounds interpretation of these data. Even at the lower concentrations of humic substance employed there was ample humic material present to saturate the surface of the mercury drop. The adsorption of humic substances on a HMDE was characterized in Chapter 7; undoubtedly these

effects were exacerbated by use of an adsorptive collection technique, especially in conjunction with DP mode! Hence, it was not possible to establish whether the decrease in the measured CSV stripping peak in the presence of humic substance was caused by association of the cobalt complex with the humic substances and/or by inhibited accumulation of the complex due to adsorbed humic substances.

9.3.3 Solvent Extraction

The stoichiometry and stability of the nonionic Zn(II) and Cd(II) complexes with PAN, and their interaction with humic substances was studied by solvent extraction. Spectroscopic measurements, in both aqueous and organic solvents, established that these complexes were formed significantly at the concentrations used. This type of experiment is ideal for studying the interaction of humic substances with metal complexes because, due to their insolubility in chloroform, humic substances are excluded from the organic phase and hence cannot interfere in the measurements.

Although stable in aqueous solution and in octanol, the $Cd(PAN)_2$ complex decomposed slowly with time in chloroform; $Zn(PAN)_2$ was stable in chloroform.

Increments of Zn(II) and PAN were added, at a constant molar ratio of 1:2.3, to solutions of humic and fulvic acids (0.1 mg mL^{-1} SHHA or 0.2 mg mL^{-1} FA4) to generate solutions in the concentration range 7.70×10^{-6} - 2.31×10^{-5} mol L^{-1} PAN, and 3.36×10^{-6} - 1.01×10^{-5} mol L^{-1} Zn(II) respectively. Relative to a 'blank' solution containing no humic substance, the absorbance for the $Zn(PAN)_2$ solution (at 555 nm) was decreased slightly in the presence of fulvic and humic acids (by *ca.* 10% at all concentrations studied). If this observation is indicative of some interaction between $Zn(PAN)_2$ and the humic substances then any effect should be enhanced at lower concentrations of the complex (albeit at the expense of sensitivity and reproducibility of data). Therefore, increments of Zn(II) and PAN were added (molar ratio 1:2.3) to a 0.1 mg mL^{-1} SHHA solution to generate solutions 1.9×10^{-6} to 5.8×10^{-6} mol L^{-1} in PAN, and 8.4×10^{-7} to 2.5×10^{-6} mol L^{-1} in Zn(II) respectively. Under these conditions, reproducibility for replicate experiments was *ca.* 10%; there was no significant difference between the amount of PAN complex extracted into the chloroform phase in the presence or absence of humic acid. The amount of humic substance present, relative to the

concentration of metal complex, was greater than that used for the visible absorption spectroscopic studies with $Cu(PAN)_2$ (Chapter 8). This indicates the weaker binding affinity of humic substances for $Zn(PAN)_2$ than for $Cu(PAN)_2$, and is consistent with hydrophilic binding to form ternary complexes, HS-M-L, as described in Chapter 8.

The humic substance was equilibrated with $Zn(PAN)_2$ in aqueous solution for 5 min prior to extraction into chloroform. Allowing equilibration to occur for several hours before addition of chloroform caused no change in the amount of extractable complex. It is important to note that there will be a certain amount of chloroform present in the aqueous phase during the extraction process, and that this may change the structure and chemical behaviour of humic substances (Johnsen, 1987).

These results do not support or exclude a hydrophobic interaction between humic substances and $Zn(PAN)_2$. The chloroform represents a competing hydrophobic phase, and is present in much greater concentration than is any hydrophobic humic phase. Therefore, any hydrophobic interaction between the humic substance and the metal complex would have to be very stable to allow detection by this technique.

It was concluded that this technique did not provide adequate sensitivity for these studies and/or that there is no significant interaction between humic substances and $Zn(PAN)_2$.

9.3.4 Equilibrium Dialysis Utilizing Radiochemical Detection

Radiotracers allow very low concentrations of metal ions to be used thus maximizing the sensitivity of a technique; they also eliminate contamination problems (although extraneous metal ions may be present, they would not be detected). Dialysis experiments reported in Chapter 5 indicated that only 11% of an SHHA solution passed through a 3 500 MWCO membrane at pH 7.3; hence, negligible amounts were expected to pass through 1 000 MWCO dialysis tubing.

Humic substance solutions were dialyzed against $Cd^{109}(PAN)_2$; any association of the humic substance with the metal complex would result in enhanced activity inside the dialysis tubing. On dialysis of 5 and 25 ppm SHHA against $Cd(PAN)_2$, the activity detected in the retentate at equilibrium was the same as that in the external solution. In contrast, for a 25 ppm FA4 solution, the activity associated with the fulvic acid solution

was twice that for the external solution, indicating some interaction. It was inferred that this indicates a *hydrophilic* interaction to form a Cd(II)-fulvic acid complex or a ternary FA-Cd-PAN complex.

In support of this hypothesis, dialysis of 25 ppm FA4 against Cd^{109}-APDC (3.21×10^{-10} mol L^{-1} Cd(II), 1.94×10^{-5} mol L^{-1} APDC; in 0.1 mol L^{-1} acetate buffer, pH 4.8) also resulted in the Cd^{109} activity of the FA4 retentate being twice that of the dialyzate. However, subsequent spectroscopic measurements indicated only very weak complexation of Cd(II) by APDC; hence, fulvic acid would be interacting predominantly with 'free' Cd(II).

The greater interaction with Cd(II), or $Cd(PAN)_2$, observed for fulvic acid than for humic acid is interesting. Results presented in Chapter 6 indicated that humic acid is the stronger hydrophilic complexor for Cu(II). However, the sulphur content of these samples may play an important role in their association with Cd(II). Analysis by ICP-MS established a sulphur content of $< 0.02\%$ for SHHA, compared with 0.69% for FA4.

Importantly, these results indicate that any *hydrophobic* interactions between humic substances and hydrophobic metal complexes are negligible at the concentrations used in this work. That is, at environmentally significant concentrations, reactions of humic substances are predominantly hydrophilic with the chelating properties of their acidic functional groups dominating over any hydrophobic associations. It is noted that, relative to compounds such as DDT, the metal complexes studied are not extremely hydrophobic (Chapter 8).

CHAPTER 10

SUMMARY

The solubility and aggregation properties of humic and fulvic acids and their interactions with hydrophobic metal complexes and aqueous metal ions have been studied.

The heterogeneity and complexity of humic substances present some unique difficulties and challenge the way in which techniques used to probe their properties are interpreted. The great environmental importance of humic substances provides the impetus for research on this complex class of materials. Humic substances interfere in the application or interpretation of many experimental techniques. These effects must be characterized and compensated for whenever possible. Undoubtedly the failure of many workers to recognize this fact has led to the wide variation in properties of humic substances reported in the literature. Further, the fact that humic substances are poorly characterized allows almost any result to be explained! Obviously future research must be directed towards resolving these discrepancies such that 'real' differences between humic samples can be distinguished from technique-dependent effects.

In the present work attempts were made to characterize, and subsequently compensate for, the nature of humic substance interferences in all techniques used. A comparative approach was adopted which allowed the change in properties between different samples, or the change in properties of one particular sample under different experimental conditions, to be assessed. Although it would be ideal, quantitative analysis of the properties of humic substances in absolute terms was not considered feasible. Complex models, such as those proposed to describe metal complexation by humic substances, which are presented as being "quantitative" may indeed be misleading.

Detailed specific structures have been proposed for humic acid; however, these are not considered relevant for such a heterogeneous material. There is evidence that the properties of humic substances (e.g. their interaction with metal ions) are more than just a simple summation over their components. Any particular property of humic substances is controlled by the collective actions of a large number of heterogeneous constituents (weighted in an unknown

way); hence, consideration of their global, macroscopic behaviour would seem the most realistic approach (Buffle, 1990).

10.1 Solubility and Aggregation Properties of Humic Acid

The solubility and aggregation properties of humic acids are fundamental to an understanding of their macromolecular structure, of their mobility in soils and natural waters, and are relevant to the development of extraction protocols.

The use of gel permeation chromatography and equilibrium dialysis confirmed the molecular size heterogeneity present in humic acid solutions in equilibrium with the solid phase and established that solubilization of larger molecules occurred with increased pH and with decreased ionic strength. These results indicated that a pH of at least 8 was required to solubilize a 'representative' humic acid sample.

In conjunction with studies on the adsorption of humic acid on XAD resins (in which size exclusion and incomplete adsorption were observed) these results highlighted the operational nature of the fraction defined as 'humic acid'. The reported differences between aquatic and soil-derived humic substances could arise in part from the different extraction methods employed. In order to obtain information which is relevant to environmental systems isolation procedures which provide the most chemically mild conditions should be favoured. Ideally, *in situ* techniques should be used for studying humic substances, but few provide adequate sensitivity.

10.2 Interaction of Humic Substances with Hydrophobic Metal Complexes

The toxicity of hydrophobic compounds to biota is well documented, and humic substances are known to ameliorate this toxicity. A parallel has been drawn between the interaction of humic acids with nonpolar compounds and the increase in water solubility of hydrophobic species by micelles in which a microscopic phase is formed through aggregation of surfactant monomers.

One aim of the present work was to investigate the possible existence of hydrophobic zones in humic acid aggregates. Hydrophobic metal complexes (CuL_2) were used as a probe. It was thought that these species could be solubilized in the 'hydrophobic core' of a humic acid 'micelle'. However, it was established that humic substances interacted hydrophilically with

these species (if necessary displacing a low molecular weight ligand) to form a ternary complex, HS-Cu-L. That is, at the concentrations of humic substance used in this work no evidence to support a 'micelle model' for the structure of humic acid was obtained. The existence of such aggregates at environmentally relevant concentrations seems unlikely. Rather, the properties of humic substances appeared to be dominated by their hydrophilic acidic functional groups.

Recently the extreme toxicity to algae of hydrophobic Cu(II) complexes (e.g. Cu-8-hydroxyquinoline) has been reported. In the present work, it was found that humic acid could ameliorate this toxicity, but only if the displaced ligand itself is not toxic. This result is environmentally important. It is apparent that the contribution from humic substances (the ligands dominating complexing in soils and natural waters) must be considered in any assessment of the impact of pollutants on a system. This highlights the complexity of environmental systems and establishes the importance of speciation measurements as opposed to (often) meaningless analysis of, say, the total metal content of a sample.

10.3 Interaction of Humic Substances with Aqueous Metal Ions

The propensity of humic substances to complex with metal ions is well documented. By use of ion selective electrode potentiometry, the present work established that humic substances form very stable Cu(II) complexes. By comparison with discrete ligands, aliphatic carboxyl moieties, e.g. malonate and citrate, were found to be the most appropriate models for humic substance chelating groups; salicylate and phthalate (although perhaps the numerically dominant moieties) complexed Cu(II) far too weakly to be considered as significant complexors in weakly-acidic to near-neutral solutions.

It is noted that this approach is simplistic. No one discrete ligand could adequately model the humic substance Cu(II) binding curves over the entire pH range (2.5 - 7.5). For the heterogeneous humic substance systems other interactions, such as aggregate formation, must be considered. Further, a lack of detailed knowledge about the structural components of humic substances affects our ability to model their properties. For example, nitrogen donors complex Cu(II) very strongly; however, nitrogen moieties in humic substances have not been completely characterized.

Another phenomenon which could contribute to the stability of humic complexes with Cu(II) is cascade binding (enhanced intramolecular coordination of weak donor groups by virtue of their proximity to strongly coordinating sites). A parallel study of Cu(II) complexation by the discrete ligand 5-methoxy-N-(2-hydroxybenzyl)sarcosine (which forms a 6-membered chelate ring on coordination with Cu(II)) illustrated the possible importance of this phenomenon.

Studies on the complexation of metal ions by humic substances provided results pertinent to an understanding of speciation in the environment. Fulvic acids are the dominant soluble ligands in soils and natural waters, whereas humic acids are more likely to be associated with the particulate phase. Normalized to carboxyl content, humic acid was found to be a much stronger Cu(II) complexor than was fulvic acid. Further, the larger humic acid moieties formed more stable Cu(II) complexes than did the smaller molecules. This may indicate that the particulate humic phase is an important source or sink for metal ions in the environment. However, it was also observed that other environmentally significant metal ions, e.g. Al(III) and Mg(II), can compete for humic Cu(II) complexation sites.

Again, this highlights the complexity of environmental systems. It indicates that results obtained for isolated humic substances with discrete metal species may be extrapolated to predict environmental conditions only with great caution - or not at all!!

The interaction of Cu(II) with humic substances also provided interesting insights into the distribution of chelating moieties in humic and fulvic acids. Assuming bidentate coordination, then for fulvic acid at pH 5.0, 6.3, and 7.0 respectively, complexation capacity measurements indicated that 82 - 85%, 67 - 72%, and 50 - 60% of the carboxyl groups were not involved in strong Cu(II) binding under the experimental conditions. For humic acid the proportions were, 73 -79 %, 33 - 43% and 5 - 25% respectively. The greater proportion of humic carboxyl groups involved in complexation indicates a more 'efficient' distribution of the fewer chelating moieties. In fulvic acid, a significant number of the carboxyl groups may be structurally isolated, stereochemically inaccessible, and/or present in tricarballylate, tetrabutanoate, or salicylate type configurations which are weak complexors below pH 7.0.

REFERENCES

ABBT-BRAUN, G., FRIMMEL, F.H. & SCHULTEN, H-R. 1989. Structural investigations of aquatic humic substances by pyrolysis-field ionization mass spectrometry and pyrolysis-gas chromatography/mass spectrometry. *Water Research*, **12**, 1579-1591.

ABDUL, A.S., GIBSON, T.L. & RAI, D.N. 1990. Use of humic acid solution to remove organic contaminants from hydrogeologic systems. *Environmental Science and Technology*, **24**, 328-333.

ABDULLAH, P.B. & MONK, C.B. 1985. Potentiometric studies of some lanthanum carboxylates at constant ionic strength. *Journal of the Chemical Society - Faraday Transactions 1*, **81**, 983-990.

ACEBAL, S.A. & REBELLO, A.D.L. 1983. Studies on the anodic stripping voltammetry of lead in polluted estuarine waters. *Analytica Chimica Acta*, **148**, 71-78.

ADAMIC, M.L. & BARTAK, D.E. 1984. Quantitation of metal complexes by reverse-pulse amperometry and molecular-exclusion chromatography. *Analytica Chimica Acta*, **158**, 43-55.

AGARWAL, S.C. & NETON, J. 1989. Mutagenicity and alkylating activity of the aqueous chlorination products of humic acid and their molecular weight fractions. *The Science of the Total Environment*, **79**, 69-83.

AHSANULLAH, M. & FLORENCE, T.M. 1984. Toxicity of copper to the marine amphipod *Allorchestes compressa* in the presence of water- and lipid-soluble ligands. *Marine Biology*, **81**, 41-45.

AIKEN, G.R., THURMAN, E.M., MALCOLM, R.L. & WALTON, H.F. 1979. Comparison of XAD macroporous resins for the concentration of fulvic acid from aqueous solution. *Analytical Chemistry*, **51**, 1799-1803.

AIKEN, G.R. 1984. Evaluation of ultrafiltration for determining molecular weight of fulvic acid. *Environmental Science and Technology*, **18**, 978-981.

AIKEN, G.R. & MALCOLM, R.L. 1987. Molecular weight of aquatic fulvic acids by vapor pressure osmometry. *Geochimica et Cosmochimica Acta*, **51**, 2177-2184.

AIKEN, G.R., BROWN, P.A., NOYES, T.I. & PICKNEY, D.J. 1989. Molecular size and weight of fulvic and humic acids from the Suwannee River. In: *Humic Substances in the Suwannee River, Georgia: Interactions, Properties, and Proposed Structures*. R.C. Averett, J.A. Leenheer, D.M. McKnight and K.A. Thorn (eds), U.S. Geological Survey Open-File Report **87-557**, pp 163-178.

ALBERTS, J.J., FILIP, Z. & LEVERSEE, G.J. 1989. Interaction of estuarine organic matter with copper and benzo(a)pyrene. *Marine Chemistry*, **28**, 77-87.

ALEKSANDROVA, L.N. 1960. The use of sodium pyrophosphate for isolating free humic substances and their organic-mineral compounds from the soil. *Soviet Soil Science*, **2**, 190-197.

ALMENDROS, G., MARTIN, F. & GONZALEZ-VILA, F.J. 1987. Depolymerization and degradation of humic acids with sodium perborates. *Geoderma*, **39**, 235-247.

ALTMANN, R.S. & BUFFLE, J. 1988. The use of differential equilibrium functions for interpretation of metal binding in complex ligand systems: its relation to site occupation and site affinity distributions. *Geochimica et Cosmochimica Acta*, **52**, 1505-1519.

AMADOR, J.A., MILNE, P.J., MOORE, C.A. & ZIKA, R.G. 1990. Extraction of chromophoric humic substances from seawater. *Marine Chemistry*, **29**, 1-17.

AMY, G.L., COLLINS, M.R., KUO, C.J. & KING, P.H. 1987. Comparing gel permeation chromatography and ultrafiltration for the molecular weight characterization of aquatic organic matter. *Journal of the American Water Works Association*, **49**, 43-49.

ANDERSON, H.A. & HEPBURN, A. 1977. Fractionation of humic acid by gel permeation chromatography. *Journal of Soil Science*, **28**, 634-644.

ANDERSON, H.A., BERROW, M.L., FARMER, V.C., HEPBURN, A., RUSSELL, J.D. & WALKER, A.D. 1982. A reassessment of the podzol formation process. *Journal of Soil Science*, **33**, 125-136.

ANSON, F.C., FLANAGAN, J.B., TAKAHASI, K. & YAMADA, A. 1976. Some virtues of differential pulse polarography in examining adsorbed reactants. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **67**, 253-259.

ANSON, F.C., NI, C-L. & SAVEANT, J-M. 1985. Electrocatalysis at redox polymer electrodes with separation of the catalytic and charge propagation roles. Reduction of O_2 to H_2O_2 as catalysed by cobalt(II) tetrakis(4-N-methylpyridyl)porphyrin. *Journal of the American Chemical Society*, **107**, 3442-3450.

ANTWORTH, C.P., YATES, R.R. & COOPER, W.T. 1989. Application of inverse chromatography in organic geochemistry -I. Characterization of polar solute - soil organic matter interactions by high performance liquid chromatography. *Organic Geochemistry*, **14**, 157-164.

APTE, S.C., GARDNER, M.J. & HUNT, D.T.E. 1989. An evaluation of dialysis as a size-based separation method for the study of trace metal speciation in natural waters. *Environmental Technology Letters*, **10**, 201-212.

APTE, S.C., GARDNER, M.J., RAVENSCROFT, J.E. & TURRELL, J.A. 1990. Examination of the range of copper complexing ligands in natural waters using a combination of cathodic stripping voltammetry and computer simulation. *Analytica Chimica Acta*, **235**, 287-297.

ATHAVALE, V.T., MAHADEVAN, N., MATHUR, P.K. & SATHE, R.M. 1967. Potentiometric study of the complexes of malonic, 5-sulphosalicylic and chromotropic acids with some metal ions. *Journal of Inorganic and Nuclear Chemistry*, **29**, 1947-1951.

ATHERTON, N.M., CRANWELL, P.A., FLOYD, A.J. & HAWORTH, R.D. 1967. Humic acid - I. ESR spectra of humic acid. *Tetrahedron*, **23**, 1653-1667.

AVDEEF, A. 1983. Weighting scheme for regression analysis using pH data from acid-base titrations. *Analytica Chimica Acta*, **148**, 237-244.

AVDEEF, A., ZABRONSKY, J. & STUTING, H.H. 1983. Calibration of copper ion selective electrode response to pCu 19. *Analytical Chemistry*, **55**, 298-304.

AVDEEF, A. 1985. STBLTY: Methods for construction and refinement of equilibrium models. In: *Computational Methods for the Determination of Formation Constants*. D.J. Legget (ed), Plenum Press, New York, pp 355-473.

AVERETT, R.C., LEENHEER, J.A., McKNIGHT, D.M. & THORN, K.A. (eds). 1989. *Humic Substances in the Suwannee River, Georgia: Interactions, Properties, and Proposed Structures*. U.S. Geological Survey Open-File Report **87-557**, Denver, Colorado.

BACKHUS, D.A. & GSCHWEND, P.M. 1980. Fluorescent polycyclic aromatic hydrocarbons as probes for studying the impact of colloids on pollutant transport in groundwater. *Environmental Science and Technology*, **24**, 1214-1223.

BAEZA, J.J.B., RAMOS, G.R. & FERNANDEZ, C.M. 1989. Comparative study of several programs used in the potentiometric evaluation of equilibrium constants including an error sensitivity analysis. *Analytica Chimica Acta*, **223**, 419-427.

BACKES, C.A. & TIPPING, E. 1987. Aluminium complexation by an aquatic humic fraction under acidic conditions. *Water Research*, **21**, 211-216.

BALLARD, T.M. 1971. Role of humic carrier substances in DDT movement through forest soil. *Soil Science Society of America Proceedings*, **35**, 145-157.

BARICA, J. 1978. Unusual response of a cupric ion electrode in prairie lake waters. *Journal. Fisheries Research Board of Canada*, **35**, 141-143.

BARKER, S.A., FINCH, P., HAYES, M.H.B., SIMMONDS, R.G. & STACEY, M. 1965. Isolation and preliminary characterization of soil polysaccharides. *Nature*, **205**, 68-69.

BARRON, P.F., WILSON, M.A., STEPHENS, J.F., CORNELL, B.A. & TATE, K.R. 1980. Cross-polarization ^{13}C NMR spectroscopy of whole soils. *Nature*, **286**, 585-587.

BARTH, H.G. 1980. A practical approach to steric exclusion chromatography of water-soluble polymers. *Journal of Chromatographic Science*, **18**, 409-429.

BARTLE, K.D., POMFRET, A., PAPPIN, A.J., MILLS, D.G. & EVLIGA, H. 1987. Analysis of methylated humic acids from fossil fuels by size exclusion chromatography and NMR. *Organic Geochemistry*, **11**, 139-149.

BASCOMB, C.L. 1968. Distribution of pyrophosphate-extractable iron and organic carbon in soils of various groups. *Journal of Soil Science*, **19**, 251-268.

BATES, R.G. 1973. *Determination of pH. Theory and Practice.* John Wiley & Sons, New York.

BATLEY, G.E. & FLORENCE, T.M. 1974. An evaluation and comparison of some techniques of anodic stripping voltammetry. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **55**, 23-43.

BATLEY, G.E. & FLORENCE, T.M. 1976a. Determination of the chemical forms of dissolved cadmium, lead and copper in seawater. *Marine Chemistry*, **4**, 347-363.

BATLEY, G.E. & FLORENCE, T.M. 1976b. The effect of dissolved organics on the stripping voltammetry of seawater. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **72**, 121-126.

BATLEY, G.E. & FARRAR, Y.J. 1978. Irradiation techniques for the release of bound heavy metals in natural waters and blood. *Analytica Chimica Acta*, **99**, 283-292.

BATLEY, G.E & GARDNER, D. 1978. A study of copper, lead and cadmium speciation in some estuarine and coastal marine waters. *Estuarine and Coastal Marine Science*, **7**, 59-70.

BAUGHMAN, G.L. & PARIS, D.F. 1981. Microbial bioconcentration of organic pollutants from aquatic systems - a critical review. *CRC Critical Reviews in Microbiology*, **8**, 205-228.

BAXTER, R.M. & CAREY, J.H. 1982. Reactions of singlet oxygen in humic waters. *Freshwater Biology*, **12**, 285-292.

BECK, M.T. 1970. *Chemistry of Complex Equilibria.* Van Nostrand Reinhold Company Ltd, London.

BECKETT, R., JUE, Z. & GIDDINGS, J.C. 1987. Determination of molecular weight distributions of fulvic and humic acids using flow field-flow fractionation. *Environmental Science and Technology*, **21**, 289-295.

BECKWITH, R.S. & NAYYAR, V.K. 1984. Extraction of soil organic matter under chemically mild conditions. *Communications in Soil Science and Plant Analysis*, **15**, 295-307.

BENGTSSON, G., ENFIELD, C.G. & LINDQVIST, R. 1987. Macromolecules facilitate the transport of trace organics. *The Science of the Total Environment*, **67**, 159-164.

BERTINO, D.J., ALBRO, P.W. & HASS, J.R. 1987. Enzymatic hydrolysis of carbohydrates in aquatic fulvic acid. *Environmental Science and Technology*, **21**, 859-863.

BETTERIDGE, D., FERNANDO, Q. & FREISER, H. 1963. Solvent extraction of certain transition metal ions with 1-(2-pyridylazo)-2-naphthol. *Analytical Chemistry*, **35**, 294-298.

BEVERIDGE, A. & PICKERING, W.F. 1984. Influence of surfactants on the determination of Cu, Pb and Cd by ASV. *Water Research*, **18**, 1119-1123.

BIEDERBECK, V.O. & PAUL, E.A. 1973. Fractionation of soil humate with phenolic solvents and purification of the nitrogen-rich portion with polyvinylpyrrolidone. *Soil Science*, **115**, 357-366.

BLOK, V.C., SLATER, P.G. & GIBLIN, E.M. 1983 Comparison of sorption and extraction methods for recovery of trace organics from water. *Water Science and Technology*, **15**, 149-159.

BLONDEAU, R. 1986a. The fractionation of humic acids on Sephadex gel: the role of salts and extractants. *Agrochimica*, **30**, 128-136.

BLONDEAU, R. 1986b. Comparison of soil humic and fulvic acids of similar molecular weight. *Organic Geochemistry*, **9**, 47-50.

BLOOMFIELD, C. 1981. The translocation of metals in soils. In: *The Chemistry of Soil Processes*. D.J. Greenland & M.H.B. Hayes (eds), Wiley-Interscience, Chichester, pp 463-504.

BLUM, D.J.W. & SPEECE, R.E. 1990. Determining chemical toxicity to aquatic species. *Environmental Science and Technology*, **24**, 284-293.

BLUST, R., VERHEYEN, E., DOUMEN, C. & DECLEIR, W. 1986. Effect of complexation by organic ligands on the bioavailability of copper to the brine shrimp *Artemia* SP. *Aquatic Toxicology*, **8**, 211-221.

BODALBHAI, L. & BRAJTER-TOTH, A. 1990. Scanning electron microscopy in the analysis of the activity of graphite electrodes. *Analytica Chimica Acta*, **231**, 191-201.

BOEHM, P.D. & QUINN, J.G. 1973. Solubilization of hydrocarbons by the dissolved organic matter in seawater. *Geochimica et Cosmochimica Acta*, **37**, 2459-2477.

BOLLAG, J-M. & LOLL, M.J. 1983. Incorporation of xenobiotics into soil humus. *Experentia*, **39**, 1221-1231.

BOND, A.M. 1980. *Modern Polarographic Methods in Analytical Chemistry*, Marcel Dekker, New York.

BONNETT, R. & COUSINS, R.P.C. 1987. On the metal content and metal ion uptake of botanically specific peats and the derived humic acids. *Organic Geochemistry*, **11**, 497-503.

BORGGAARD, O.K. 1974. Titrimetric determination of acidity and pK values of humic acid. *Acta Chemica Scandinavica*, **A28**, 121-122.

BOTTARI, E., LIBERTI, A. & RUFOLO, A. 1969. On the formation of Cu^{II}-tartrate complexes in acid solution. *Inorganic Chimica Acta*, **3**, 201-206.

BOTTERO, J.Y., CASES, J.M., FIESSINGER, F. & POIRIER, J.E. 1980. Studies of hydrolyzed aluminum chloride solutions. 1. Nature of aluminum species and composition of aqueous solutions. *Journal of Physical Chemistry*, **84**, 2933-2939.

BOUDOU, A., GEORGESCAULD, D. & DESMAZES, J.P. 1983. Exotoxicological role of the membrane barriers in transport and bioaccumulation of mercury compounds. *Advances in Environmental Science and Technology*, **13**, 117-136.

BRACEWELL, J.M., ROBERTSON, G.W. & WELCH, D.I. 1980. Polycarboxylic acids as the origin of some pyrolysis products characteristic of soil organic matter. *Journal of Analytical and Applied Pyrolysis*, **2**, 239-248.

BRAIBANTI, A., DALLAVALLE, F., MORI, G. & VERONI, B. 1982. Analysis of variance applied to determinations of equilibrium constants. *Talanta*, **29**, 725-731.

BRAIBANTI, A., OSTACOLI, G., PAOLETTI, P., PETTIT, L.D. & SAMMARTANO, S. 1987. Recommended procedure for testing the potentiometric apparatus and technique for the pH-metric measurement of metal-complex equilibrium constants. *Pure and Applied Chemistry*, **59**, 1721-1728.

BRAJTER, K., OLBRYCH-SLESZYNSKA, E. & STASKIEWICZ, M. 1988. Preconcentration and separation of metal ions by means of Amberlite XAD-2 loaded with pyrocatechol violet. *Talanta*, **35**, 65-67.

BREMNER, J.M. 1949. Studies on soil organic matter. Part II. The extraction of organic carbon and nitrogen from soil. *Journal of Agricultural Science*, **39**, 280-282.

BREMNER, J.M. & LEES, H. 1949. Studies on soil organic matter. Part II. The extraction of organic matter from soil by neutral reagents. *Journal of Agricultural Science*, **39**, 274-279.

BREMNER, J.M. 1950. Some observations on the oxidation of soil organic matter in the presence of alkali. *Journal of Soil Science*, **1**, 198-204.

BRESNAHAN, W.T., GRANT, C.L. & WEBER, J.H. 1978. Stability constants for the complexation of copper(II) ions with water and soil fulvic acids measured by an ion selective electrode. *Analytical Chemistry*, **50**, 1675-1679.

BREZONIK, P.L., BRAUNER, P.A. & STUMM, W. 1976. Trace metal analysis by anodic stripping voltammetry: effect of sorption by natural and model organic compounds. *Water Research*, **10**, 605-612.

BRIHAYE, C., GILLAIN, G. & DUYCKAERTS, G. 1983. Determination of traces of metals by anodic stripping voltammetry at a rotating glassy carbon ring-disc electrode. Part 3. Evaluation of linear anodic stripping voltammetry with ring collection for the determination of cadmium, lead and copper in pure water and high-purity sodium chloride, and of cadmium, lead, copper, antimony and bismuth in seawater. *Analytica Chimica Acta*, **148**, 51-57.

BRISBANE, P.G., AMATO, M. & LADD, J.N. 1972. Gas chromatography analysis of amino acids from the action of proteolytic enzymes on soil humic acids. *Soil Biology and Biochemistry*, **4**, 51-61.

BROOK, A.J.W. & HOUSLEY, S. 1969. Interaction of phenols with Sephadex gels. *Journal of Chromatography*, **41**, 200-204.

BROWN, P.A. & LEENHEER, J.A. 1989. Significance of density determination in molecular structures comprising fulvic acid from the Suwannee River. In: *Humic Substances in the Suwannee River, Georgia: Interactions, Properties, and Proposed Structures*. R.C. Averett, J.A. Leenheer, D.M. McKnight & K.A. Thorn (eds), U.S. Geological Survey Open-File Report, **87-557**, pp 311-330.

BROWN, P.L., SYLVA, R.N., BATLEY, G.E. & ELLIS, J. 1985. The hydrolysis of metal ions. Part 8. Aluminium(III). *Journal of the Chemical Society - Dalton Transactions*, 1967-1970.

BUDDRUS, J., BURBA, P., HERZOG, H. & LAMBERT, J. 1989. Quantitation of partial structures of aquatic humic substances by one- and two-dimensional solution ^{13}C nuclear magnetic resonance spectroscopy. *Analytical Chemistry*, **61**, 628-631.

BUFFLE, J., GRETER, F.-L., NEMBRINI, G., PAUL, J. & HAERDI, W. 1976. Capabilities of voltammetric techniques for water quality control problems. *Fresenius Zeitschrift für Analytische Chemie*, **282**, 339-350.

BUFFLE, J., GRETER, F.-L. & HAERDI, W. 1977. Measurement of complexation properties of humic and fulvic acids in natural waters with lead and copper ion-selective electrodes. *Analytical Chemistry*, **49**, 216-222.

BUFFLE, J., COMINOLI, A., GRETER, F.-L. & HAERDI, W. 1978a. Voltammetric behaviour of humic and fulvic substances of natural waters. *Proceedings - Analytical Division of the Chemical Society*, **15**, 59-61.

BUFFLE, J., DELADOEY, P. & HAERDI, W. 1978b. The use of ultrafiltration for the separation and fractionation of organic ligands in freshwaters. *Analytica Chimica Acta*, **101**, 339-357.

BUFFLE, J. & GRETER, F.-L. 1979. Voltammetric study of humic and fulvic substances. Part II. Mechanism of reaction of the Pb-fulvic complexes on the mercury electrode. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **101**, 231-251.

BUFFLE, J. 1980. A critical comparison of studies of complex formation between copper(II) and fulvic substances of natural waters. *Analytica Chimica Acta*, **118**, 29-44.

BUFFLE, J., DELADOEY, P., GRETER, F.-L. & HAERDI, W. 1980. Study of the complex formation of copper(II) by humic and fulvic substances. *Analytica Chimica Acta*, **116**, 255-274.

BUFFLE, J. 1981. Calculation of the surface concentration of the oxidised metal during the stripping step in the anodic stripping techniques and its influence on speciation measurements in natural waters. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **125**, 273-294.

BUFFLE, J. & COMINOLI, A. 1981. Voltammetric study of humic and fulvic substances. Part IV. Behaviour of fulvic substances at the mercury-water interface. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **121**, 273-299.

BUFFLE, J. 1984. Natural organic matter and metal-organic interactions in aquatic systems. *Metal Ions in Biological Systems*, **18**, 165-221.

BUFFLE, J. & STAUB, C. 1984. Measurement of complexation properties of metal ions in natural conditions by ultrafiltration: measurement of equilibrium constants for complexation of zinc by synthetic and natural ligands. *Analytical Chemistry*, **56**, 2837-2842.

BUFFLE, J., TESSIER, A. & HAERDI, W. 1984. Interpretation of trace metal complexation by aquatic organic matter. In: *Complexation of Trace Metals in Natural Waters*. C.J.M. Kramer & J.C. Duinker (eds), Martinus Nijhoff/Dr W. Junk, The Hague, pp 301-316.

BUFFLE, J. & ALTMANN, R.S. 1987. Interpretation of metal complexation by heterogeneous complexants. In: *Aquatic Surface Chemistry*. W. Stumm (ed), Wiley-Interscience, New York, pp 351-383.

BUFFLE, J., MOTA, A.M. & GONCALVES, M.L.S. 1987a. Adsorption of fulvic-like organic ligands and their Cd and Pb complexes at a mercury electrode. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **223**, 235-262.

BUFFLE, J., VUILLEUMIER, J.J., TERCIER, M.L. & PARTHASARATHY, N. 1987b. Voltammetric study of humic and fulvic substances. V. Interpretation of metal ion complexation measured by anodic stripping voltammetric methods. *The Science of the Total Environment*, **60**, 75-96.

BUFFLE, J. 1988. *Complexation Reactions in Aquatic Systems: An Analytical Approach*. Ellis Horwood Ltd, Chichester.

BUFFLE, J. 1990. The analytical challenge posed by fulvic and humic compounds. *Analytica Chimica Acta*, **232**, 1-2.

BUFFLE, J., ALTMANN, R.S., FILELLA, M. & TESSIER, A. 1990a. Complexation by natural heterogeneous compounds: site occupation distribution functions, a normalized description of metal complexation. *Geochimica et Cosmochimica Acta*, **54**, 1535-1553.

BUFFLE, J., ALTMANN, R.S. & FILELLA, M. 1990b. Effect of physico-chemical heterogeneity of natural complexants. Part II. Buffering action and role of their background sites. *Analytica Chimica Acta*, **232**, 225-237.

BURCH, R.D., LANGFORD, C.H. & GAMBLE, D.S. 1978. Methods for the comparison of fulvic acid samples: the effects of origin and concentration on acidic properties. *Canadian Journal of Chemistry*, **56**, 1196-1201.

BURNHAM, A.K., CALDER, G.V., FRITZ, J.S., JUNK, G.A., SVEC, H.J. & WILLIS, R. 1972. Identification and estimation of neutral organic contaminants in potable water. *Analytical Chemistry*, **44**, 139-142.

BUSING, W.R. & LEVY, H.A. 1962. ORGLS, a general FORTRAN least squares program. *Oak Ridge National Laboratory Report*, **ORNL-TM-271**.

BUTLER, J.H.A. & LADD, J.N. 1969. Effect of extractant and molecular size on the optical and chemical properties of soil humic acids. *Australian Journal of Soil Research*, **7**, 229-239.

BUTTRY, D.A. & ANSON, F.C. 1983. Effects of electron exchange and single-file diffusion on charge propagation in Nafion films containing redox couples. *Journal of the American Chemical Society*, **105**, 685-689.

CABANISS, S.E., SHUMAN, M.S. & COLLINS, B.J. 1984. Metal-organic binding: a comparison of models. In: *Complexation of Trace Metals in Natural Waters*. C.J.M. Kramer & J.C. Duinker (eds), Martinus Nijhoff/Dr W. Junk, The Hague, pp 165-179.

CABANISS, S.E. & SHUMAN, M.S. 1986. Combined ion selective electrode and fluorescence quenching detection for copper-dissolved organic matter titrations. *Analytical Chemistry*, **58**, 398-401.

CABANISS, S.E. & SHUMAN, M.S. 1988a. Copper binding by dissolved organic matter: I. Suwannee River fulvic acid equilibria. *Geochimica et Cosmochimica Acta*, **52**, 185-193.

CABANISS, S.E. & SHUMAN, M.S. 1988b. Copper binding by dissolved organic matter: II. Variation in type and source of organic matter. *Geochimica et Cosmochimica Acta*, **52**, 195-200.

CABANISS, S.E. & SHUMAN, M.S. 1988c. Fluorescence quenching measurements of copper-fulvic acid binding. *Analytical Chemistry*, **60**, 2418-2421.

CABANISS, S.E., MOREL, F.M.M. & MARINSKY, J.A. 1989. Comment on "A unified physicochemical description of the protonation and metal ion complexation equilibria of natural organic acids (humic and fulvic acids)". *Environmental Science and Technology*, **23**, 746-747.

CABANISS, S.E. 1990. pH and ionic strength effects on nickel-fulvic acid dissociation kinetics. *Environmental Science and Technology*, **24**, 583-588.

CABBINESS, D.K. & MARGERUM, D.W. 1970. Effect of macrocyclic structures on the rate of formation and dissociation of copper(II) complexes. *Journal of the American Chemical Society*, **92**, 2151-2153.

CACECI, M.S. & BILLON, A. 1990. Evidence for large organic scatterers (50 - 200 nm diameter) in humic acid samples. *Organic Geochemistry*, **15**, 335-350.

CALLAWAY, J.Y., GABBITA, K.V. & VILKER, V.L. 1984. Reduction of low molecular weight halocarbons in the vapor phase above concentrated humic acid solutions. *Environmental Science and Technology*, **18**, 890-893.

CAMERON, R.S., SWIFT, R.S., THORNTON, B.K. & POSNER, A.M. 1972a. Calibration of gel permeation chromatography materials for use with humic acid. *Journal of Soil Science*, **23**, 342-349.

CAMERON, R.S., THORNTON, B.K., SWIFT, R.S. & PRESTON, A.M. 1972b. Molecular weight and shape of humic acid from sedimentation and diffusion measurements on fractionated extracts. *Journal of Soil Science*, **23**, 394-403.

CAMPANELLA, L. 1987. Discussion on "Electroanalysis of heavy metal/organic matter systems" by R.F.M.J. Cleven, P.M. Wolfs & H.P. van Leeuwen. In: *Metals Speciation, Separation, and Recovery*, J.W. Patterson & R. Passino (eds), Lewis Publishers, Michigan, pp 260-264.

CAMPI, E., OSTACOLI, G., MEIRONE, M. & SAINI, G. 1964. Stability of the complexes of tricarballylic and citric acids with bivalent metal ions in aqueous solution. *Journal of Inorganic and Nuclear Chemistry*, **26**, 553-564.

CAPONE, S., DE ROBERTIS, A., DE STEFANO, C., SAMMARTANO, S. & SCARCELLA, R. 1985. Ionic strength dependence of formation constants. Part 7. Protonation constants of low molecular weight carboxylic acids at 10, 25 and 45°C. *Thermochimica Acta*, **86**, 273-280.

CARLBERG, G.E. & MARTINSEN, K. 1982. Adsorption/complexation of organic micropollutants to aquatic humus. Influence of aquatic humus with time on organic pollutants and comparison of two analytical methods for analysing organic pollutants in humus water. *The Science of the Total Environment*, **25**, 245-254.

CARON, G., SUFFET, I.H. & BELTON, T. 1985. Effect of dissolved organic carbon on the environmental distribution of nonpolar organic compounds. *Chemosphere*, **14**, 993-1 000.

CARON, G. & SUFFET, I.H. 1989. Binding of nonpolar pollutants to dissolved organic carbon. In: *Aquatic Humic Substances. Influence on Fate and Treatment of Pollutants*. I.H. Suffet & P. MacCarthy (eds), American Chemical Society, Advances in Chemistry Series 219, Washington DC, pp 117-130.

CARTER, C.W. & SUFFET, I.H. 1982. Binding of DDT to dissolved humic materials. *Environmental Science and Technology*, **16**, 735-740.

CAVALLARO, N. & McBRIDE, M.B. 1980. Response of the Cu^{2+} and Cd^{2+} ion-selective electrodes to solutions of different ionic strength and ion composition. *Soil Science Society of America Journal*, **44**, 881-882.

CHEAM, V. 1973. Chelation study of copper(II):fulvic acid system. *Canadian Journal of Soil Science*, **53**, 377-382.

CHEAM, V. & GAMBLE, D.S. 1974. Metal-fulvic acid chelation equilibrium in aqueous $NaNO_3$ solution. Hg(II), Cd(II), and Cu(II) fulvate complexes. *Canadian Journal of Soil Science*, **54**, 413-417.

CHEN, Y. & SCHNITZER, M. 1976a. Scanning electron microscopy of a humic acid and of a fulvic acid and its metal and clay complexes. *Soil Science Society of America Journal*, **40**, 682-686.

CHEN, Y. & SCHNITZER, M. 1976b. Viscosity measurements on soil humic substances. *Soil Science Society of America Journal*, **40**, 866-872.

CHEN, Y., SENESI, N. & SCHNITZER, M. 1977. Information provided on humic substances by E_4/E_6 ratios. *Soil Science Society of America Journal*, **41**, 352-358.

CHESHIRE, M.V., CRANWELL, P.A., FALSHAW, C.P., FLOYD, A.J. & HAWORTH, R.D. 1967. Humic acid - II. Structure of humic acids. *Tetrahedron*, **23**, 1669-1682.

CHESHIRE, M.V., BERROW, M.L., GOODMAN, B.A. & MUNDIE, C.M. 1977. Metal distribution and nature of some Cu, Mn and V complexes in humic and fulvic acid fractions of soil organic matter. *Geochimica et Cosmochimica Acta*, **41**, 1131-1138.

CHIAVARI, G., PASTORELLI, L. & VITALI, P. 1984. Analytical concentration of phenolic compounds from water solutions. *Fresenius Zeitschrift für Analytische Chemie*, **317**, 130-131.

CHIN, Y-P. & WEBER, W.J. Jr. 1989. Estimating the effects of dispersed organic polymers on the sorption of contaminants by natural solids. 1. A predictive thermodynamic humic substance - organic solute interaction model. *Environmental Science and Technology*, **23**, 978-984.

CHIOU, C.T., PETERS, L.J. & FREED, V.H. 1979. A physical concept of soil-water equilibria for nonionic organic compounds. *Science*, **206**, 831-832.

CHIOU, C.T., PETERS, L.J. & FREED, V.H. 1981. Soil-water equilibriums for nonionic organic compounds. Reply to comments. *Science*, **213**, 684.

CHIOU, C.T., SCHMEDDING, D.W. & MANES, M. 1982. Partitioning of organic compounds in octanol-water systems. *Environmental Science and Technology*, **16**, 4-10.

CHIOU, C.T., PORTER, P.E. & SCHMEDDING, D.W. 1983. Partition equilibria of nonionic organic compounds between soil organic matter and water. *Environmental Science and Technology*, **17**, 227-231.

CHIOU, C.T. 1985. Partition coefficients of organic compounds in lipid-water systems and correlations with fish bioconcentration factors. *Environmental Science and Technology*, **19**, 57-62.

CHIOU, C.T. & SHOUP, T.D. 1985. Soil sorption of organic vapors and effects of humidity on sorptive mechanism and capacity. *Environmental Science and Technology*, **19**, 1196-1200.

CHIOU, C.T., SHOUP, T.D. & PORTER, P.E. 1985. Mechanistic roles of soil humus and minerals in the sorption of nonionic organic compounds from aqueous and organic solutions. *Organic Geochemistry*, **8**, 9-14.

CHIOU, C.T. & MANES, M. 1986. Application of the Flory-Huggins theory to the solubility of solids in glyceryl trioleate. *Journal of the Chemical Society - Faraday Transactions*, **82**, 243-246.

CHIOU, C.T., MALCOLM, R.L., BRINTON, T.I. & KILE, D.E. 1986. Water solubility enhancement of some organic pollutants and pesticides by dissolved humic and fulvic acids. *Environmental Science and Technology*, **20**, 502-508.

CHIOU, C.T., KILE, D.E., BRINTON, T.I., MALCOLM, R.L., LEENHEER, J.A. & MacCARTHY, P. 1987. A comparison of water solubility enhancements of organic solutes by aquatic humic materials and commercial humic acids. *Environmental Science and Technology*, **21**, 1231-1234.

CHIOU, C.T., KILE, D.E. & MALCOLM, R.L. 1988. Sorption of vapors of some organic liquids on soil humic acid and its relation to partitioning of organic compounds in soil organic matter. *Environmental Science and Technology*, **22**, 298-303.

CHIOU, C.T., LEE, J-F. & BOYD, S.A. 1990. The surface area of soil organic matter. *Environmental Science and Technology*, **24**, 1164-1166.

CHOPPIN, G.R. & KULLBERG, L. 1978. Protonation thermodynamics of humic acid. *Journal of Inorganic and Nuclear Chemistry*, **40**, 651-654.

CHOUDHRI, M.B. & STEVENSON, F. 1957. Chemical and physicochemical properties of soil humic colloids. III. Extraction of organic matter form soils. *Soil Science Society of America Proceedings*, **21**, 508-513.

CLEVEN, R.F.M.J., DE JONG, H.G. & VAN LEEUWEN, H.P. 1986. Pulse polarography of metal/polyelectrolyte complexes and operation of the mean diffusion coefficient. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **202**, 57-68.

CLEVEN, R.F.M.J., WOLFS, P.M. & VAN LEEUWEN, H.P. 1987. Electroanalysis of heavy metal/organic matter systems. In: *Metals Speciation, Separation, and Recovery*. J.W. Patterson & R. Passino (eds), Lewis Publishers, Michigan, pp 243-259.

CLEVEN, R.F.M.J., DEL CASTILHO, P. & WOLFS, P.M. 1988. Effects of organic matter on the pulse voltammetric speciation of copper. *Environmental Technology Letters*, **9**, 869-876.

COALE, K.H. & BRULAND, K.W. 1988. Copper complexation in the Northeast Pacific. *Limnology and Oceanography*, **33**, 1084-1101.

COLLAZO-LOPEZ, H., YATES, R.R. & COOPER, W.T. 1989. Application of inverse chromatography in organic geochemistry -II. Measurement of solute activity coefficients in organic geopolymers by gas chromatography. *Organic Geochemistry*, **14**, 165-170.

COLLINS, M.R., AMY, G.L. & STEELINK, C. 1986. Molecular weight distribution, carboxylic acidity, and humic substances content of aquatic organic matter: implications for removal during water treatment. *Environmental Science and Technology*, **20**, 1028-1032.

COMINOLI, A., BUFFLE, J. & HAERDI, W. 1980. Voltammetric study of humic and fulvic substances. Part III. Comparison of the capabilities of the various polarographic techniques for the analysis of humic and fulvic substances. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **110**, 259-275.

CONNELL, D.W. 1988. Bioaccumulation behaviour of persistent organic chemicals with aquatic organisms. *Reviews of Environmental Contamination and Toxicology*, **101**, 117-154.

COOPER, W.J., ZIKA, R.G., PETASNE, R.G. & PLANE, J.M.C. 1988. Photochemical formation of H_2O_2 in natural waters exposed to sunlight. *Environmental Science and Technology*, **22**, 1156-1160.

CORSINI, A., YIH, I.M-L., FERNANDO, Q. & FREISER, H. 1962. Potentiometric investigation of the metal complexes of 1-(2-pyridylazo)-2-naphthol and 4-(2-pyridylazo)-resorcinol. *Analytical Chemistry*, **34**, 1090-1093.

COSOVIC, B. 1985. Aqueous surface chemistry: assessment of adsorption characteristics of organic solutes by electrochemical methods. In: *Chemical Processes in Lakes*. W. Stumm (ed), John Wiley & Sons, New York, pp 55-80.

COSOVIC, B. & VOJVODIC, V. 1987. Direct determination of surface active substances in natural waters. *Marine Chemistry*, **22**, 363-373.

COVINGTON, A.K. 1981. Recent developments in pH standardization and measurement for dilute aqueous solutions. *Analytica Chimica Acta*, **127**, 1-21.

COVINGTON, A.K., BATES, R.G. & DURST, R.A. 1985. Definition of pH scales, standard reference values, measurement of pH and related terminology. *Pure and Applied Chemistry*, **57**, 531-542.

CRESSEY, P.J., MONK, G.R., POWELL, H.K.J. & TENNENT, D.J. 1983. Fulvic acid studies: evidence for a polycarboxylate co-ordination mode at soil pH. *Journal of Soil Science*, **34**, 783-799.

CRISPONI, G., NURCHI, V., GANADA, M.L. & LUBINU, G. 1990. Potentiometric and ^{13}C NMR study of the interaction between boric acid and pyrogallol (1,2,3-trihydroxybenzene). *Polyhedron*, **9**, 789-793.

CROOK, E.H., McDONNELL, R.P. & McNULTY, J.T. 1975. Removal and recovery of phenols from industrial waste effluents with Amberlite XAD polymeric adsorbents. *Industrial and Engineering Chemistry Product Research and Development*, **14**, 113-118.

DAIGNAULT, S.A., NOOT, D.K., WILLIAMS, D.T. & HUCK, P.M. 1988. A review of the use of XAD resins to concentrate organic compounds in water. *Water Research*, **22**, 803-813.

DANIELE, P.G., RIGANO, C. & SAMMARTANO, S. 1982. Ionic strength dependence of formation constants. Part II: Potentiometric study of the copper(II)-malonate-glycinate system in the range $0.01 \leq I \leq 1.0$. *Transition Metal Chemistry*, **7**, 109-112.

DANIELE, P.G., RIGANO, C. & SAMMARTANO, S. 1983. The formation of proton and alkali metal complexes with ligands of biological interest in aqueous solution. Thermodynamics of Li^+, Na^+ and K^+-dicarboxylate complex formation. *Thermochimica Acta*, **62**, 101-112.

DANIELE, P.G., RIGANO, C. & SAMMARTANO, S. 1985a. Ionic strength dependence of formation constants. Alkali metal complexes of ethylenediaminetetraacetate, nitriloacetate, diphosphate, and tripolyphosphate in aqueous solution. *Analytical Chemistry*, **57**, 2956-2960.

DANIELE, P.G., DE ROBERTIS, A., DE STEFANO, C., SAMMARTANO, S. & RIGANO, C. 1985b. On the possibility of determining the thermodynamic parameters for the formation of weak complexes using a simple model for the dependence on ionic strength of activity coefficients: Na^+, K^+ and Ca^{2+} complexes of low molecular weight ligands in aqueous solution. *Journal of the Chemical Society - Dalton Transactions*, 2353-2361.

DAVIES, C.W. 1962. *Ion Association*. Butterworth, London.

DAVIS, H. & MOTT, C.J.B. 1981. Titrations of fulvic acid fractions. I: Interactions influencing the dissociation/reprotonation equilibria. *Journal of Soil Science*, **32**, 379-391

DAVIS, J.A. & LECKIE, J.O. 1978. Effect of adsorbed complexing ligands on trace metal uptake by hydrous oxides. *Environmental Science and Technology*, **12**, 1309-1315.

DAVIS, J.A. 1982. Adsorption of natural dissolved organic matter at the oxide/water interface. *Geochimica et Cosmochimica Acta*, **46**, 2381-2393.

DAVIS, J.A. 1984. Complexation of trace metals by adsorbed organic matter. *Geochimica et Cosmochimica Acta*, **48**, 679-691.

DAVIES, R.P. & DOBBS, A.J. 1984. The prediction of bioconcentration in fish. *Water Research*, **18**, 1253-1262.

DAVISON, W. 1978. Defining the electroanalytically measured species in a natural water sample. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **87**, 395-404.

DEBRECZENI, F., POLGÁR, J. & NAGYPÁL, I. 1983. NMR relaxation studies in solutions of transition metal complexes. VIII. Equilibrium dynamics in aqueous solution of copper(II)-N-methylethylenediamine, -N,N'-dimethylethylenediamine and -N-methylglycine systems. *Inorganica Chimica Acta*, **71**, 195-200.

DEBYE, P. & HUCKEL, E. 1923. The theory of electrolytes. I. Lowering of the freezing point and related phenomena. *Physikalische Zeitschrift*, **24**, 185-206.

DEC, J., SHUTTLEWORTH, K.L. & BOLLAG, J.-M., 1990. Microbial release of 2,4-dichlorophenol bound to humic acid or incorporated during humification. *Journal of Environmental Quality*, **19**, 546-551.

DE HAAN, H., WERLEMARK, G. & DE BOER, T. 1983. Effect of pH on molecular weight and size of fulvic acids in drainage water from peaty grassland in NW Netherlands. *Plant and Soil*, **75**, 63-73.

DE KIMPE, C.R. & SCHNITZER, M. 1990. Low-temperature ashing of humic and fulvic acid. *Soil Science Society of America Journal*, **54**, 399-403.

DELAHAY, P. 1965. *Double Layer and Electrode Kinetics*. John Wiley & Sons, New York.

DELL'AGNOLA, G. & FERRARI, G. 1971. Molecular sizes and functional groups of humic substances extracted by 0.1 M pyrophosphate from soil aggregates of different stability. *Journal of Soil Science*, **22**, 342-349.

DEMETRIOU, J.A., MACIAS, R.F.M., McARTHUR, M.J. & BEATTIE, J.M. 1968. Gel filtration chromatography of fluorescent phenolic and heterocyclic compounds. *Journal of Chromatography*, **34**, 342-350.

DEMPSEY, B.A. & O'MELIA, C.R. 1983. Proton and calcium complexation of four fulvic acid fractions. In: *Aquatic and Terrestrial Humic Materials*. R.F. Christman & E.T. Gjessing (eds), Ann Arbor Science, Ann Arbor, pp 239-273.

DEREPPE, J-M., MOREAUX, C. & DEBYSER, Y. 1980. Investigation of marine and terrestrial humic substances by ^{1}H and ^{13}C nuclear magnetic resonance and infrared spectroscopy. *Organic Geochemistry*, **2**, 117-124.

DE WIT, J.C.M., RIEMSDIJK, W.H., NEDERLOF, M.M., KINNIBURGH, D.G. & KOOPAL, L.K. 1990. Analysis of ion binding on humic substances and the determination of intrinsic affinity distributions. *Analytica Chimica Acta*, **232**, 189-207.

DOBBS, J.C., SUSETYO, W., CARREIRA, L.A. & AZARRAGA, L.V. 1989a. Competitive binding of protons and metal ions in humic substances by lanthanide ion probe spectroscopy. *Analytical Chemistry*, **61**, 1519-1524.

DOBBS, J.C., SUSETYO, W., KNIGHT, F.E., CASTLES, M.A., CARREIRA, L.A. & AZARRAGA, L.V. 1989b. A novel approach to metal-humic complexation studies by lanthanide ion probe spectroscopy. *International Journal of Environmental Analytical Chemistry*, **37**, 1-17.

DONAT, J.R., STATHAM, P.J. & BRULAND, K.W. 1986. An evaluation of a C-18 solid phase extraction technique for isolating metal-organic complexes from central North Pacific ocean waters. *Marine Chemistry*, **18**, 85-99.

DONG, S. & WANG, Y. 1988a. Anodic stripping voltammetric determination of trace lead with a Nafion/crown-ether film electrode. *Talanta*, **35**, 819-821.

DONG, S. & WANG, Y. 1988b. A Nafion/crown ether film electrode and its application in the anodic stripping voltammetric determination of traces of silver. *Analytica Chimica Acta*, **212**, 341-347.

DORMAAR, J.F., METCHE, M. & JACQUIN, F. 1970. Extraction and purification of humic acids from a Rendzina Ah and a Podzol Bh horizon. *Soil Biology and Biochemistry*, **2**, 285-293.

DORMAAR, J.F. 1972. Chemical properties of organic matter extracted from a number of Ah horizons by a number of methods. *Canadian Journal of Soil Science*, **52**, 67-77.

DRISCOLL, C.T. & SCHECHER, W.D. 1990. The chemistry of aluminum in the environment. *Environmental Geochemistry and Health*, **12**, 28-49.

DUBACH, P. & MEHTA, N.C. 1963. The chemistry of soil humic substances. *Soils and Fertilizers*, **26**, 293-300.

DUBIN, P.L. & PRINCIPI, J.M. 1989. Hydrophobicity parameter for aqueous size exclusion chromatography gels. *Analytical Chemistry*, **61**, 780-781.

DURST, R.A., KOCH, W.F. & WU, Y.C. 1987. pH theory and measurement. *Ion-Selective Electrode Reviews*, **9**, 173-196.

DUTT, N.K., GUPTA, S. & NAG, K. 1976. Stability constants of the complexes of Al(III), Ga(III), In(III), Fe(III) & Cr(III) with malonic & substituted malonic acids. *Indian Journal of Chemistry*, **14A**, 1000-1003.

DZOMBAK, D.A., FISH, W. & MOREL, F.M.M. 1986. Metal-humate interactions. 1. Discrete ligand and continuous distribution models. *Environmental Science and Technology*, **20**, 669-675.

EADIE, B.J., MOREHEAD, N.R. & LANDRUM, P.F. 1990. Three-phase partitioning of hydrophobic organic compounds in Great Lakes waters. *Chemosphere*, **20**, 161-178.

EISNER, U. & MARK, H.B. Jr. 1970. The anodic stripping voltammetry of trace silver solutions employing graphite electrodes. Application to silver analysis of rain and snow samples from silver iodide seeded clouds. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **24**, 345-355.

ELLIS, W.D. 1973. Anodic stripping voltammetry. *Journal of Chemical Education*, **50**, A131-A147.

EL-REHAILI, A.M. & WEBER, W.J. Jr. 1987. Correlation of humic substance trihalomethane formation potential and adsorption behavior to molecular weight distribution in raw and chemically treated waters. *Water Research*, **21**, 573-582.

ELWORTHY, P.H., FLORENCE, A.T. & MacFARLANE, C.B. 1968. *Solubilization by Surface-Active Agents*. Chapman & Hall, London.

ENFIELD, C.G. 1985. Chemical transport facilitated by multiphase flow systems. *Water Science and Technology*, **17**, 1-12.

ENFIELD, C.G. & BENGTSSON, G. 1988. Macromolecular transport of hydrophobic contaminants in aqueous environments. *Ground Water*, **26**, 64-70.

ENFIELD, C.G. , BENGTSSON, g. & LINDQVIST, R. 1989. Influence of macromolecules on chemical transport. *Environmental Science and Technology*, **23**, 1278-1286.

ENGSTROM, R.C. & STRASSER, V.A. 1984. Characterization of electrochemically pretreated glassy carbon electrodes. *Analytical Chemistry*, **56**, 136-141.

EPHRAIM, J.H., ALEGRET, S., MATHUTHU, A., BICKING, M., MALCOLM, R.L. & MARINSKY, J.A. 1986. A unified physicochemical description of the protonation and metal ion complexation equilibria of natural organic acids (humic and fulvic acids). 2. Influence of polyelectrolytic properties and functional group heterogeneity on the protonation equilibria of fulvic acid. *Environmental Science and Technology*, **20**, 354-366.

EPHRAIM, J.H., MARINSKY, J.A. & CRAMER, S.J. 1989. Complex-forming properties of natural organic acids. Fulvic acid complexes with cobalt, zinc and europium. *Talanta*, **36**, 437-443.

ERTEL, J.R. & HEDGES, J.I. 1984. The lignin component of humic substances: distribution among soil and sedimentary humic, fulvic, and base-insoluble fractions. *Geochimica et Cosmochimica Acta*, **48**, 2065-2074.

ERTEL, J.R. & HEDGES, J.I. 1985. Sources of sedimentary humic substances: vascular plant debris. *Geochimica et Cosmochimica Acta*, **49**, 2097-2107.

ESTEBAN, M., DE JONG, H.G. & VAN LEEUWEN, H.P. 1990. Metal speciation in polyelectrolytic systems by differential pulse anodic stripping voltammetry. *International Journal of Environmental Analytical Chemistry*, **38**, 75-83.

EVANS, H.E., EVANS, R.D. & LINGARD, S.M. 1989. Factors affecting the variation in the average molecular weight of dissolved organic carbon in freshwaters. *The Science of the Total Environment,* **81/82**, 297-306.

EVANS, L.T. 1959. The use of chelating reagents and alkaline solutions in soil organic-matter extraction. *Journal of Soil Science,* **10**, 110-118.

FARDY, J.J. & SYLVA, R.N. 1978. SIAS, a computer program for the generalized calculation of speciation in mixed metal-ligand aqueous systems. *Report Australian Atomic Energy Commission,* **AAEC/E445**, 1-20.

FARMER, V.C., SKJEMSTAD, J.O. & THOMPSON, C.H. 1983. Genesis of humus B horizons in hydromorphic humus Podzols. *Nature,* **304**, 342-344.

FARMER, V.C. & PISANIELLO, D.L. 1985. Against an aromatic structure for soil fulvic acid. *Nature,* **313**, 474-475.

FAULKNER, L.R. 1984. Chemical microstructures on electrodes. *Chemical and Engineering News,* **62**, 28-45.

FAUST, B.C. & HOIGNÉ, J. 1987. Sensitized photooxidation of phenols by fulvic acid and in natural waters. *Environmental Science and Technology,* **21**, 957-964.

FERRARI, G. & DELL'AGNOLA, G. 1963. Fractionation of the organic matter of soil by gel filtration through Sephadex. *Soil Science,* **96**, 418-421.

FIELD, T.B., McCOURT, J.L. & McBRYDE, W.A.E. 1974. Composition and stability of iron and copper citrate complexes in aqueous solution. *Canadian Journal of Chemistry,* **52**, 3119-3124.

FISCHER, R.B. & PETERS, D.G. 1970. *Chemical Equilibrium.* W.B. Saunders Company, Philadelphia.

FISCHER, W.R. 1986. Properties of and heavy metal complexation by aqueous humic extracts. *Zeitschrift für Pflanzenernahrung und Bodenkunde,* **149**, 382-399.

FISH, W. & MOREL, F.M.M. 1985a. Propagation of error in fulvic acid titration data: a comparison of three analytical methods. *Canadian Journal of Chemistry,* **63**, 1185-1193.

FISH, W. & MOREL, F.M.M. 1985b. Propagation of error in fulvic acid titration data: a comparison of three analytical methods. *Organic Geochemistry,* **8**, 119-120.

FISH, W., DZOMBAK, D.A. & MOREL, F.M.M. 1986. Metal-humate interactions. 2. Application and comparison of models. *Environmental Science and Technology,* **20**, 676-683.

FITCH, A. & STEVENSON, F.J. 1984. Comparison of models for determining stability constants of metal complexes with humic substances. *Soil Science Society of America Journal,* **48**, 1044-1050.

FITCH, A., STEVENSON, F.J. & CHEN, Y. 1986. Complexation of Cu(II) with a soil humic acid: response characteristics of the Cu(II) ion-selective electrode and ligand concentration effects. *Organic Geochemistry,* **9**, 109-116.

FLORENCE, T.M. 1970. Anodic stripping voltammetry with a glassy carbon electrode mercury-plated *in situ. Journal of Electroanalytical Chemistry and Interfacial Electrochemistry,* **27**, 273-281.

FLORENCE, T.M. & BATLEY, G.E. 1976. Trace metal species in sea-water - I. Removal of trace metals from sea-water by a chelating resin. *Talanta,* **23**, 179-186.

FLORENCE, T.M. & BATLEY, G.E. 1980. Chemical speciation in natural waters. *CRC Critical Reviews in Analytical Chemistry,* **9**, 219-296.

FLORENCE, T.M. 1980. Comparison of linear scan and differential pulse anodic stripping voltammetry at a thin mercury film glassy carbon electrode. *Analytica Chimica Acta,* **119**, 217-223.

FLORENCE, T.M. 1982. The speciation of trace elements in waters. *Talanta,* **29**, 345-364.

FLORENCE, T.M. 1983. Trace element speciation and aquatic toxicology. *Trends in Analytical Chemistry*, **2**, 162-166.

FLORENCE, T.M., LUMSDEN, B.G., & FARDY, J.J. 1983. Evaluation of some physico-chemical techniques for the determination of the fraction of dissolved copper toxic to the marine diatom *Nitzschia closterium*. *Analytica Chimica Acta*, **151**, 281-295.

FLORENCE, T.M. 1984. Recent advances in stripping analysis. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **168**, 207-218.

FLORENCE, T.M., LUMSDEN, B.G. & FARDY, J.J. 1984. Algae as indicators of copper speciation. In: *Complexation of Trace Metals in Natural Waters*. C.J.M. Kramer & J.C. Duinker (eds), Martinus Nijhoff/Dr W. Junk Publishers, The Hague, pp 411-418.

FLORENCE, T.M., STAUBER, J.L. & MANN, K.J. 1985. The reaction of copper-2,9-dimethyl-1,10-phenanthroline with hydrogen peroxide. *Journal of Inorganic Biochemistry*, **24**, 243-254.

FLORENCE, T.M. 1986. Electrochemical approaches to trace element speciation in waters. A review. *Analyst*, **111**, 489-505.

FLORENCE, T.M. & STAUBER, J.L. 1986. Toxicity of copper complexes to the marine diatom, *Nitzschia closterium*. *Aquatic Toxicology*, **8**, 11-26.

FLORENCE, T.M. & BATLEY, G. 1988. Chemical speciation and trace element toxicity. *Chemistry in Australia*, **55**, 363-366.

FLORENCE, T.M. 1989. Electrochemical techniques for trace element speciation in waters. In: *Trace Element Speciation: Analytical Methods and Problems*. G.E. Batley (ed), CRC Press, Florida, pp 77-116.

FOLSOM, B.R., POPESCU, N.A. & WOOD, J.M. 1986. Comparative study of aluminium and copper transport and toxicity in an acid-tolerant freshwater green alga. *Environmental Science and Technology*, **20**, 616-620.

FOX, L.E. 1983. Geochemistry of humic acid during estuarine mixing. In: *Aquatic and Terrestrial Humic Materials*, R.F. Christman & E.T. Gjessing (eds), Ann Arbor Science, Ann Arbor, Michigan, pp 407-427.

FRESCO, J. & FREISER, H. 1964. Distribution coefficients of certain 8-quinolinols and their copper chelates. *Analytical Chemistry*, **36**, 631-633.

FRIMMEL, F.H., BAUER, H., PUTZIEN, J., MURASECCO, P. & BRAUN, A.M. 1987. Laser flash photolysis of dissolved aquatic humic material and the sensitized production of singlet oxygen. *Environmental Science and Technology*, **21**, 541-545.

FU, P.L.K. & SYMONS, J.M. 1989. Mechanistic interactions of aquatic organic substances with anion-exchange resins. In: *Aquatic Humic Substances. Influence on Fate and Treatment of Pollutants*. I.H. Suffet & P. MacCarthy (eds), Advances in Chemistry Series Vol 219, American Chemical Society, Washington DC, pp 797-811.

FUNAHASHI, S., YAMADA, S. & TANAKA, M. 1971. Kinetics and mechanism of the ligand-substitution reaction of the copper(II)-(ethylene glycol)bis(2-aminoethyl ether)-N,N,N',N'-tetraacetate complex with 4-(2-pyridylazo)resorcinol with special reference to the effect of alkaline earth metal ions. *Inorganic Chemistry*, **10**, 257-263.

GADEL, F. & BRUCHET, A. 1987. Application of pyrolysis-gas chromatography-mass spectrometry to the characterization of humic substances resulting from decay of aquatic plants in sediments and waters. *Water Research*, **21**, 1195-1206.

GAMBLE, D.S. 1970. Titration curves of fulvic acid: the analytical chemistry of a weak acid electrolyte. *Canadian Journal of Chemistry*, **48**, 2662-2669.

GAMBLE, D.S. 1972. Potentiometric titration of fulvic acid: equivalence point calculations and acidic functional groups. *Canadian Journal of Chemistry*, **50**, 2680-2690.

GAMBLE, D.S., LANGFORD, C.H. & TONG, J.P.K. 1976. The structure and equilibria of a manganese(II) complex of fulvic acid studied by ion exchange and nuclear magnetic resonance. *Canadian Journal of Chemistry*, **54**, 1239-1245.

GAMBLE, D.S., LANGFORD, C.H. & UNDERDOWN, A.W. 1985. Light scattering measurements of Cu(II)-fulvic acid complexing: the interdependence of apparent complexing capacity and aggregation. *Organic Geochemistry*, **8**, 35-39.

GAMBLE, D.S. & LANGFORD, C.H. 1988. Complexing equilibria in mixed ligand systems: tests of theory with computer simulations. *Environmental Science and Technology*, **22**, 1325-1336.

GANELINA, E. SH. 1964. A cupric hydroxide crystal hydrate and its thermodynamic characteristics. *Zhurnal Prikladnoi Khimii*, **37**, 1358-1361.

GANS, P., SABATINI, A. & VACCA, A. 1985. Superquad: An improved general program for computation of formation constants from potentiometric data. *Journal of the Chemical Society - Dalton Transactions*, **6**, 1195-1200.

GARBARINI, D.R. & LION, L.W. 1986. Influence of the nature of soil organics on the sorption of toluene and trichloroethylene. *Environmental Science and Technology*, **20**, 1263-1269.

GAUTHIER, T.D., SHANE, E.C., GUERIN, W.F., SEITZ, W.R. & GRANT, C.L. 1986. Fluorescence quenching method for determining equilibrium constants for polycyclic aromatic hydrocarbons binding to dissolved humic materials. *Environmental Science and Technology*, **20**, 1162-1166.

GAUTHIER, T.D., SEITZ, W.R. & GRANT, C.L. 1987. Effects of structural and compositional variations of humic materials on pyrene K_{OC} values. *Environmental Science and Technology*, **21**, 243-248.

GELOTTE, B. 1960. Studies on gel filtration. *Journal of Chromatography*, **3**, 303-342.

GHASSEMI, M. & CHRISTMAN, R. 1968. Properties of the yellow organic acids of natural waters. *Limnology and Oceanography*, **13**, 583-597

GHOSH, K. & SCHNITZER, M. 1979. UV and visible absorption spectroscopic investigations in relation to macromolecular characteristics of humic substances. *Journal of Soil Science*, **30**, 735-745.

GHOSH, K. & SCHNITZER, M. 1980. Macromolecular structures of humic substances. *Soil Science*, **129**, 266-276.

GIESY, J.P., LEVERSEE, G.T. & WILLIAMS, D.R. 1977. Effects of naturally occurring aquatic organic fractions on cadmium toxicity to *Simocephalus serrulatas* (Daphnidae) and *Gambusia affinis* (Poecillidae). *Water Research*, **11**, 1013-1020.

GIESY, J.P., ALBERTS, J.J. & EVANS, D.W. 1986. Conditional stability constants and binding capacities for copper(II) by dissolved organic carbon isolated from surface waters of the southeastern United States. *Environmental Toxicology and Chemistry*, **5**, 139-154.

GILBERT, M.J.M., POWELL, H.K.J. & FARDY, J.J. 1988. Determination of cobalt in plant digests by adsorption stripping voltammetry. *Analytica Chimica Acta*, **207**, 103-109.

GJESSING, E.T. & LEE, G.F. 1967. Fractionation of organic matter in natural waters on Sephadex columns. *Environmental Science and Technology*, **1**, 631-638.

GJESSING, E.T. & BERGLIND, L.1981. Adsorption of PAH to aquatic humus. *Archiv für Hydrobiologie*, **92**, 24-30.

GOBAS, F.A.P.C., OPPERHUIZEN, A. & HUTZINGER, O.1986. Bioconcentration of hydrophobic chemicals in fish: relationship with membrane permeation. *Environmental Toxicology and Chemistry*, **5**, 637-646.

GOH, K.M. & REID, M.R. 1975. Molecular weight distribution of soil organic matter as affected by acid pre-treatment and fractionation into humic and fulvic acids. *Journal of Soil Science*, **26**, 207-222.

GOLUB, D., SOFFER, A. & OREN, Y. 1989. The electrical double layer of carbon and graphite electrodes. Part V. Specific interactions with simple ions. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **260**, 383-392.

GÓMEZ-BELINCHÓN, J.I., GRIMALT, J.O. & ALBAIGÉS, J. 1988. Intercomparison study of liquid-liquid extraction and adsorption on polyurethane and Amberlite XAD-2 for the analysis of hydrocarbons, polychlorobiphenyls, and fatty acids dissolved in seawater. *Environmental Science and Technology*, **22**, 677-685.

GONCALVES, M.L.S. & MOTA, A.M. 1987. Complexes of vanadyl and uranyl ions with the chelating groups of humic matter. *Talanta*, **34**, 839-847.

GONZALEZ-VILA, F.J., LÜDEMANN, H.-D. & MARTIN, F. 1983. ^{13}C-NMR structural features of soil humic acids and their methylated, hydrolyzed and extracted derivatives. *Geoderma*, **31**, 3-15.

GOODMAN, B.A. & CHESHIRE, M.V. 1973. Electron paramagnetic resonance evidence that copper is complexed in humic acid by the nitrogen of porphyrin groups. *Nature*, **244**, 158-159.

GOODMAN, B.A. & CHESHIRE, M.V. 1976. The occurrence of copper-porphyrin complexes in soil humic acids. *Journal of Soil Science*, **27**, 337-347.

GOODMAN, B.A. & CHESHIRE, M.V. 1979. A Mössbauer spectroscopic study of the effect of pH on the reaction between iron and humic acid in aqueous media. *Journal of Soil Science*, **30**, 85-91.

GRATHWOHL, P. 1990. Influence of organic matter from soils and sediments from various origins on the sorption of some chlorinated aliphatic hydrocarbons: implications on K_{oc} correlations. *Environmental Science and Technology*, **24**, 1687-1693.

GREENLAND, D.J. 1971. Interactions between humic and fulvic acids and clays. *Soil Science*, **111**, 34-41.

GREGOR, H.P., LATTINGER, L.B. & LOEBL, E.M. 1955. Metal-polyelectrolyte complexes. I. The polyacrylic acid-copper complex. *Journal of Physical Chemistry*, **59**, 34-39.

GREGOR, J.E. & POWELL, H.K.J. 1986a. Acid pyrophosphate extraction of soil fulvic acids. *Journal of Soil Science*, **37**, 577-585.

GREGOR, J.E. & POWELL, H.K.J. 1986b. Aluminium(III)-citrate complexes: a potentiometric and ^{13}C N.M.R. study. *Australian Journal of Chemistry*, **39**, 1851-1864.

GREGOR, J.E. & POWELL, H.K.J. 1987. Effects of extraction procedures on fulvic acid properties. *The Science of the Total Environment*, **62**, 3-12.

GREGOR, J.E. 1987. *Metal organic complexing in soil systems.* Ph. D. thesis, University of Canterbury, New Zealand.

GREGOR, J.E. & POWELL,H.K.J. 1988a. Application of sampled-d.c. anodic stripping voltammetry to metal/fulvic acid equilibria. *Analytica Chimica Acta*, **211**, 141-154.

GREGOR, J.E. & POWELL, H.K.J. 1988b. Protonation reactions of fulvic acids. *Journal of Soil Science*, **39**, 243-252.

GREGOR, J.E., POWELL, H.K.J. & TOWN, R.M. 1989a. Evidence for aliphatic mixed mode co-ordination in copper(II)-fulvic acid complexes. *Journal of Soil Science*, **40**, 661-673.

GREGOR, J.E., POWELL, H.K.J. & TOWN, R.M. 1989b. Metal-fulvic acid complexing: evidence supporting an aliphatic carboxylate mode of coordination. *The Science of the Total Environment*, **81/82**, 597-606.

GUGGENHEIM, E.A. 1935. Specific thermodynamic properties of aqueous solutions of strong electrolytes. *Philosophical Magazine*, **19**, 588-643.

GUILLARD, R.R.L. & RYTHER, J.H. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervaceae* (Cleve) Gran. *Canadian Journal of Microbiology*, **8**, 229-239.

GUNN, A.M., HUNT, D.T.E. & WINNARD, D.A. 1986. Aluminium speciation and its effect on toxicity. In: *Proceedings of the International Conference on Chemicals in the Environment.* J.N. Lester, R. Perry & R.M. Sterritt (eds), Selper, London, pp 628-635.

GUSTAFSON, R.L., ALBRIGHT, R.L., HEISLER, J., LIRIO, J.A. & REID, O.T. Jr. 1968. Adsorption of organic species by high surface area styrene-divinylbenzene copolymers. *Industrial and Engineering Chemistry Product Research and Development*, **7**, 107-115.

GUSTAFSON, R.L. & PALEOS, J. 1971. Interactions responsible for the selective adsorption of organics on organic surfaces. In: *Organic Compounds in Aquatic Environments.* S.D. Faust & J.V. Hunter (eds), Marcel Dekker, New York, pp 213-237.

GUY, R.D. & CHAKRABARTI, C.L. 1976. Studies of metal-organic interactions in model systems pertaining to natural waters. *Canadian Journal of Chemistry*, **54**, 2600-2611.

GUY, R.D. & NAMARATNE, S. 1987. Nafion-coated mercury-coated glassy carbon electrodes for metals analysis and speciation. *Canadian Journal of Chemistry*, **65**, 1133-1138.

HAAG, W.R., HOIGNÉ, J., GASSMAN, E. & BRAUN, A.M. 1984. Singlet oxygen in surface waters - Part II: Quantum yields of its production by some natural humic materials as a function of wavelength. *Chemosphere*, **13**, 641-650.

HAAS, C.N. & KAPLAN, B.M. 1985. Toluene-humic acid association equilibria: isopiestic measurements. *Environmental Science and Technology*, **19**, 643-645.

HADDAD, P.R. & HUCKENBERG, A.L. 1983. Some modern approaches to the chromatographic determination of inorganic anions. *Chemistry in Australia*, **50**, 275-278.

HADDAD, P.R. & HUCKENBERG, A.L. 1984. Determination of inorganic anions by high-performance liquid chromatography. *Journal of Chromatography*, **300**, 357-394.

HALL, K.J. & LEE, G.F. 1974. Molecular size and spectral characterization of organic matter in a meromictic lake. *Water Research*, **8**, 239-251.

HAMER, W.J. & ACREE, S.F. 1939. Potentiometric method for the accurate measurement of hydrogen-ion activity. *Journal of Research. National Bureau of Standards*, **23**, 647-662.

HAMER, W.J., BURTON, J.O. & ACREE, S.F. 1940. Second ionization constant and related thermodynamic quantities for malonic acid from 0° to 60°C. *Journal of Research. National Bureau of Standards*, **24**, 269-292.

HAMER, W.J. & ACREE, S.F. 1945. Second dissociation constant of *o*-phthalic acid and related pH values of phthalate buffers from 0° to 60°C. *Journal of Research. National Bureau of Standards*, **35**, 381-416.

HAMER, W.J., PINCHING, G.D. & ACREE, S.F. 1945. First dissociation constant of *o*-phthalic acid and related pH values of phthalate buffers from 0° to 60°C. *Journal of Research. National Bureau of Standards*, **35**, 539-564.

HANCK, K.W. & DILLARD, J.W. 1977. Determination of the complexing capacity of natural water by cobalt(III) complexation. *Analytical Chemistry*, **49**, 404-409.

HANSEN, A.M., LECKIE, J.O., MANDELL, E.F. & ALTMANN, R.S. 1990. Study of copper(II) association with dissolved organic matter in surface waters of three Mexican coastal lagoons. *Environmental Science and Technology*, **24**, 683-688.

HARNED, H.S. & OWEN, B.B. 1958. *The Physical Chemistry of Electrolytic Solutions.* Reinhold, New York.

HART, B.T. 1981. Trace metal complexing capacity of natural waters: a review. *Environmental Technology Letters*, **2**, 95-110.

HASSETT, J.P. & ANDERSON, M.A. 1979. Association of hydrophobic organic compounds with dissolved organic matter in aquatic systems. *Environmental Science and Technology*, **13**, 1526-1529.

HASSETT, J.P. & MILICIC, E. 1985. Determination of equilibrium and rate constants for binding of a polychlorinated biphenyl congener by dissolved humic substances. *Environmental Science and Technology*, **19**, 638-643.

HATCHER, P.G., VANDERHART, D.L. & EARL, W.L. 1980a. Use of solid-state ^{13}C NMR in structural studies of humic acids and humin from Holocene sediments. *Organic Geochemistry*, **2**, 87-92.

HATCHER, P.G., ROWAN, R. & MATTINGLY, M.A. 1980b. ^{1}H and ^{13}C NMR of marine humic acids. *Organic Geochemistry*, **2**, 77-85.

HATCHER, P.G., SCHNITZER, M., DENNIS, L.W. & MACIEL, G.E. 1981a. Aromaticity of humic substances in soils. *Soil Science Society of America Journal*, **45**, 1089-1094.

HATCHER, P.G., MACIEL, G.E. & DENNIS, L.W. 1981b. Aliphatic structure of humic acids: a clue to their origin. *Organic Geochemistry*, **3**, 43-48.

HATCHER, P.G., BREGER, I.A., DENNIS, L.W. & MACIEL, G.E. 1983. Solid-state ^{13}C-NMR of sedimentary humic substances: new revelations on their chemical composition. In: *Aquatic and Terrestrial Humic Materials*. R.F. Christman & E.T. Gjessing (eds), Ann Arbor Science, Ann Arbor, pp 37-81.

HATCHER, P.G., SCHNITZER, M., VASSALLO, A.M. & WILSON, M.A. 1989. The chemical structure of highly aromatic humic acids in three volcanic ash soils as determined by dipolar dephasing NMR studies. *Geochimica et Cosmochimica Acta*, **53**, 125-130.

HAUMAIER, L., ZECH, W. & FRANKE, G. 1990. Gel permeation chromatography of water-soluble organic matter with deionized water as eluent - I. Examination of the method. *Organic Geochemistry*, **15**, 413-417.

HAWORTH, R.D. 1971. The chemical nature of humic acid. *Soil Science*, **111**, 71-79.

HAYANO, S., SHINOZUKA, N. & HYAKUTAKE, M. 1982. Surface active properties of marine humic acids. *Journal. Japan Oil Chemists' Society*, **31**, 357-362.

HAYASE, K. & TSUBOTA, H. 1983. Sedimentary humic acid and fulvic acid as surface active substances. *Geochimica et Cosmochimica Acta*, **47**, 947-952.

HAYES, M.H.B. 1970. Adsorption of triazine herbicides on soil organic matter, including a short review on soil organic matter chemistry. *Residue Reviews*, **32**, 131-174.

HAYES, M.H.B. & SWIFT, R.S. 1978. The chemistry of soil organic colloids. In: *The Chemistry of Soil Constituents*. D.J. Greenland & M.H.B. Hayes (eds), Wiley, Chichester, pp 179-320.

HAYES, M.H.B. 1985. Extraction of humic substances from soil. In: *Humic Substances in Soil, Sediment, and Water: Geochemistry, Isolation, and Characterization*. G.R. Aiken, D.M. McKnight, R.L. Wershaw & P. MacCarthy (eds), Wiley-Interscience, New York, pp 329-362.

HEDLUND, T., SJÖBERG, S. & ÖHMAN, L-O. 1987. Equilibrium and structural studies of silicon(IV) and aluminium(III) in aqueous solution. 15. A potentiometric study of speciation and equilibria in the Al^{3+}-$CO_2(g)$-OH^- system. *Acta Chemica Scandinavica*, **A41**, 197-207.

HEDWIG, G.R. & POWELL, H.K.J. 1971. Direct potentiometric measurement of hydrogen ion concentrations in sodium chloride solutions of fixed ionic strength. *Analytical Chemistry*, **43**, 1206-1212.

HEDWIG, G.R. 1972. *Complexes with Aliphatic Oximes, Ketones and Amines. A Thermodynamic Study*. Ph. D. thesis, University of Canterbury, New Zealand.

HEFFORD, R.J.W. & PETTIT, L.D. 1981. Potentiometric and spectrophotometric study of the co-ordination compounds formed between copper(II) and dipeptides containing tyrosine. *Journal of the Chemical Society - Dalton Transactions*, 1331-1335.

HEIJNE, G.J.M. & VAN DER LINDEN, W.E. 1978. The formation of mixed copper sulfide-silver sulfide membranes for copper(II)-selective electrodes. Part III. The electrode response in the presence of complexing agents. *Analytica Chimica Acta*, **96**, 13-22.

HEJZLAR, J. 1987. Effect of inorganic salts and adsorption in Sephadex-gel chromatography of aquatic organic substances. *Water Research*, **21**, 1311-1318.

HELLIWELL, S., BATLEY, G.E., FLORENCE, T.M. & LUMSDEN, B.G. 1983. Speciation and toxicity of aluminium in a model fresh water. *Environmental Technology Letters*, **4**, 141-144.

HERBES, S.E., SOUTHWORTH, G.R. & GEHRS, C.W. 1976. Organic contaminants in aqueous coal conversion effluents: environmental consequences and research priorities. *Trace Substances in Environmental Health*, **10**, 295-303.

HERING, J.G., SUNDA, W.G., FERGUSON, R.L. & MOREL, F.M.M. 1987. A field comparison of two methods for the determination of copper complexation: bacterial bioassay and fixed potential amperometry. *Marine Chemistry*, **20**, 299-312.

HERING, J.G. & MOREL, F.M.M. 1988a. Humic acid complexation of calcium and copper. *Environmental Science and Technology*, **22**, 1234-1237.

HERING, J.G. & MOREL, F.M.M. 1988b. Kinetics of trace metal complexation: role of alkaline-earth metals. *Environmental Science and Technology*, **22**, 1469-1478.

HERING, J.G. & MOREL, F.M.M. 1989. Slow coordination reactions in seawater. *Geochimica et Cosmochimica Acta*, **53**, 611-618.

HINE, P.T. & BURSILL, D.B. 1984. Gel permeation chromatography of humic acid. Problems associated with Sephadex gel. *Water Research*, **18**, 1461-1465.

HIRAIDE, M., ARIMA, Y. & MIZUIKE, A. 1987. Separation and determination of traces of heavy metals complexed with humic substances in river waters by sorption on indium-treated Amberlite XAD-2 resin. *Analytica Chimica Acta*, **200**, 171-179.

HIRATA, S. 1981. Stability constants for the complexes of transition-metal ions with fulvic and humic acids in sediments measured by gel filtration. *Talanta*, **28**, 809-815.

HIROSE, K. 1990. Chemical speciation of trace metals in seawater: implication of particulate trace metals. *Marine Chemistry*, **28**, 267-274.

HOFFMANN, M.R., YOST, E.C., EISENREICH, S.J. & MAIER, W.J. 1981. Characterization of soluble and colloidal-phase metal complexes in river water by ultrafiltration. A mass-balance approach. *Environmental Science and Technology*, **15**, 655-661.

HOLLAND, H.D. 1978. *The Chemistry of the Atmosphere and Oceans*, John Wiley & Sons, New York.

HOOGVLIET, J.C., VAN DEN BELD, C.M.B., VAN DER POEL, C.J. & VAN BENNEKOM, W.P. 1986. Influence of polishing and of electrochemical pretreatment on the performance of glassy-carbon electrodes in electrochemical detection. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **201**, 11-21.

HOYER, B., FLORENCE, T.M. & BATLEY, G.E. 1987. Application of polymer-coated glassy carbon electrodes in anodic stripping voltammetry. *Analytical Chemistry*, **59**, 1608-1614.

HOYER, B. & FLORENCE, T.M. 1987. Application of polymer-coated glassy carbon electrodes to the direct determination of trace metals in body fluids by anodic stripping voltammetry. *Analytical Chemistry*, **59**, 2839-2842.

HUCKINS, J.N., TUBERGEN, M.W. & MANUWEERA, G.K. 1990. Semipermeable membrane devices containing model lipid: a new approach to monitoring the bioavailability of lipophilic contaminants and estimating their bioconcentration potential. *Chemosphere*, **20**, 533-552.

HUFFMAN, E.W.D.Jr. & STUBER, H.A. 1985. Analytical methodology for elemental analysis of humic substances. In: *Humic Substances in Soil, Sediment, and Water. Geochemistry, Isolation, and Characterization.* G.R. Aiken, D.M. McKnight, R.L. Wershaw & P. MacCarthy (eds), Wiley-Interscience, New York, pp 433-455.

HUIZENGA, D.L. & PATTERSON, H.H. 1988. A chemiluminescence technique for determination of rates of reaction of chromium(III) with carboxylate ligands and humic acid. *Analytica Chimica Acta*, **206**, 263-272.

HUME, D.N. & CARTER, J.N. 1972. Characteristics of the mercury coated graphite electrode in anodic stripping voltammetry: application to the study of trace metals in environmental water systems. *Chemia Analityczna*, **17**, 747-759.

HUNTER, K.A. & LEE, K.C. 1986. Polarographic study of the interaction between humic acids and other surface-active organics in river waters. *Water Research*, **20**, 1489-1491.

IKAN, R., IOSELIS, P., RUBINSZTAIN, Y., AIZENSHTAT, Z., PUGMIRE, R., ANDERSON, L.L. & ISHIWATARI, R. 1986a. Carbohydrate origin of humic substances. *Naturwissenschaften*, **73**, 150-151.

IKAN, R., RUBINSZTAIN, Y., IOSELIS, P., AIZENSHTAT, Z., PUGMIRE, R., ANDERSON, L.L. & WOOLFENDEN, W.R. 1986b. Carbon-13 cross polarized magic-angle samples spinning nuclear magnetic resonance of melanoidins. *Organic Geochemistry*, **9**, 199-212.

INGRI, N. & SILLEN, L.G. 1964. High-speed computers as a supplement to graphical methods. IV. An ALGOL version of LETAGROPVRID. *Arkiv foer Kemi*, **23**, 97-121.

INOUE, K., ZHAO, L.P. & HUANG, P.M. 1990. Adsorption of humic substances by hydroxyaluminum- and hydroxyaluminosilicate-montmorillonite complexes. *Soil Science Society of America Journal*, **54**, 1166-1172.

IRVING, H.M., MILES, M.G. & PETTIT, L.D. 1967. A study of some problems in determining the stoichiometric proton dissociation constants of complexes by potentiometric titrations using a glass electrode. *Analytica Chimica Acta*, **38**, 475-488.

ISHIWATARI, R. 1973. Chemical characterization of fractionated humic acids from lake and marine sediments. *Chemical Geology*, **12**, 113-126.

ISHIWATARI, R., HAMANA, H. & MACHIHARA, T. 1980. Isolation and characterization of polymeric organic materials in a polluted river water. *Water Research*, **14**, 1257-1262.

ITALIA, M.P. & UDEN, P.C. 1988. Multiple element emission spectral detection gas chromatographic profiles of halogenated products from chlorination of humic acid and drinking water. *Journal of Chromatography*, **438**, 35-43.

IWAMOTO, T. 1961. Acid-base property and metal chelate formation of 4-(2-pyridylazo)resorcinol. *Bulletin. Chemical Society of Japan*, **34**, 605-610.

JACKSON, G.E. & COSGROVE, A. 1982. Studies on the chelation of aluminium for biological application. Part 2. Oxalic, malonic, and succinic acids. *Suid-Afrikaanse Tydskrif vir Chemie*, **35**, 93-95.

JACOBS, J.S.A., CHRISTMAN, R.F. & JOHNSON, J.D. 1988. High-pressure size-exclusion chromatography of chlorinated and unchlorinated aquatic fulvic acids using polyacrylamide gel. *Journal of Chromatography*, **450**, 433-442.

JACOBSEN, E. & LINDSETH, H. 1976. Effects of surfactants in differential pulse polarography. *Analytica Chimica Acta*, **86**, 123-127.

JANSON, J-C. 1967. Adsorption phenomena on Sephadex. *Journal of Chromatography*, **28**, 12-20.

JARDIM, W.F. & ALLEN, H.E. 1984. Measurement of copper complexation by naturally occurring ligands. In: *Complexation of Trace Metals in Natural Waters*. C.J.M. Kramer & J.C. Duinker (eds), Martinus Nijhoff/ Dr W. Junk, The Hague, pp 1-15.

JARDINE, P.M., WEBER, N.L. & McCARTHY, J.F. 1989. Mechanisms of dissolved organic carbon adsorption on soil. *Soil Science Society of America Journal*, **53**, 1378-1385.

JOHNSEN, S. 1987. Interactions between polycyclic aromatic hydrocarbons and natural aquatic humic substances. Contact time relationship. *The Science of the Total Environment*, **67**, 269-278.

JOLLY, W.J. (ed). 1968. *Inorganic Syntheses*. Vol XI, McGra-Hill, U.S.A.

JUNK, G.A., RICHARD, J.J., GRIESER, M.D., WITIAK, D., WITIAK, J.L., ARGUELLO, M.D., VICK, R., SVEC, H.J., FRITZ, J.S. & CALDER, G.V. 1974. Use of macroreticular resins in the analysis of water for trace organic contaminants. *Journal of Chromatography*, **99**, 745-762.

JUNK, G.A. & RICHARD, J.J. 1988. Organics in water: solid phase extraction on a small scale. *Analytical Chemistry*, **60**, 451-454.

KALINOWSKI, E. & BLONDEAU, R. 1988. Characterization of sedimentary humic acids fractionated by hydrophobic interaction chromatography. *Marine Chemistry*, **24**, 29-37.

KALLIANOU, C.S., YASSOGLOU, N.J. & ZIECHMANN, W. 1987. Characterization of humic substances obtained from calcerous soils from Greece with various extractants. II. Chemical characterization. *Zeitschrift für Pflanzenernahrung Dungung und Bodenkunde*, **150**, 108-112.

KAMAU, G.N. 1988. Surface preparation of glassy carbon electrodes. *Analytica Chimica Acta*, **207**, 1-16.

KATEMAN, G., SMIT, H.C. & MEITES, L. 1983. Weighting in the interpretation of data for potentiometric acid-base titrations by non-linear regression. *Analytica Chimica Acta*, **152**, 61-72.

KEMP, A.L.W. & WONG, H.K.T. 1974. Molecular-weight distribution of humic substances from Lakes Ontario and Erie sediments. *Chemical Geology*, **14**, 15-22.

KENNEDY, J.A. 1984. *Metal-Organic Complexing in Natural Soil Systems*. Ph. D. Thesis, University of Canterbury, New Zealand.

KENNEDY, J.A., POWELL, H.K.J. & TAYLOR, M.C. 1983. *O*-phthalic acid as a standard substance for calibrations of hydrogen ion concentrations with a glass electrode. *Analytica Chimica Acta*, **147**, 351-357.

KENNEDY, J.A. & POWELL, H.K.J. 1985. Polyphenol interactions with aluminium(III) and iron(III): their possible involvement in the podzolization process. *Australian Journal of Chemistry*, **38**, 879-888.

KEPLEY, L.J. & BARD, A.J. 1988. Ellipsometric, electrochemical, and elemental characterization of the surface phase produced on glassy carbon electrodes by electrochemical activation. *Analytical Chemistry*, **60**, 1459-1467.

KILE, D.E. & CHIOU, C.T. 1989a. Water-solubility enhancement of nonionic organic contaminants. In: *Aquatic Humic Substances. Influence on Fate and Treatment of Pollutants*, I.H. Suffet & P. MacCarthy (eds), American Chemical Society, Advances in Chemistry Series 219, Washington DC, pp 131-157.

KILE, D.E. & CHIOU, C.T. 1989b. Water-solubility enhancements of DDT and trichlorobenzene by some surfactants below and above the critical micelle concentration. *Environmental Science and Technology*, **23**, 832-838.

KILE, D.E., CHIOU, C.T. & BRINTON, T.I. 1989. Interactions of organic contaminants with fulvic and humic acids from the Suwannee River and other humic substances in aqueous systems, with inferences to the structures of humic molecules. In: *Humic Substances in the Suwannee River, Georgia: Interactions, Properties, and Proposed Structures*. R.C. Averett, J.A. Leenheer, D.M. McKnight and K.A. Thorn (eds), U.S. Geological Survey Open-File Report **87-557**, pp 39-57.

KILE, D.E., CHIOU, C.T. & HELBURN, R.S. 1990. The effect of some petroleum sulfonate surfactants on the apparent water solubility of organic compounds. *Environmental Science and Technology*, **24**, 205-208.

KIM, J.I., BUCKAU, G., LI, G.H., DUSCHNER, H. & PSARROS, N. 1990. Characterization of humic and fulvic acids from Gorleben groundwater. *Fresenius Journal of Analytical Chemistry*, **338**, 245-252.

KO, W.H. & LOCKWOOD, J.L. 1968. Accumulation and concentration of chlorinated hydrocarbon pesticides by microorganisms in soil. *Canadian Journal of Microbiology*, **14**, 1075-1078.

KOUNAVES, S.P. & BUFFLE, J. 1988. An iridium based mercury film electrode. Part II. Comparison of mercury-film behaviors: theory versus reality. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **239**, 113-123.

KRAMER, C.J.M., GUO-HUI, Y., & DUINKER, J.C. 1984. Possibilities for misinterpretation in ASV-speciation studies of natural waters. *Fresenius Zeitschrift für Analytische Chemie*, **317**, 383-384.

KREMLING, K., WENCK, A. & OSTERROHT, C. 1981. Investigations on dissolved copper-organic substances in Baltic waters. *Marine Chemistry*, **10**, 209-219.

KREMMER, T. & BOROSS, L. 1979. *Gel Chromatography: Theory, Methodology, Applications.* Wiley-Interscience, Hungary.

KRONBERG, L., HOLMBOM, B., REUNANEN, M. & TIKKANEN, L. 1988. Identification and quantification of the Ames mutagenic compound 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone and of its geometric isomer (E)-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid in chlorine-treated humic water and drinking water extracts. *Environmental Science and Technology*, **22**, 1097-1103.

KRUCK, T.P.A., BOYD, A., ORVIG, C. & CRAPPER McCLACHLAN, D.R. 1990. Aluminium as a pathogen: ligand dependent neurotoxic effects of dietary aluminium. submitted to *The Lancet.*

KRZNARIC, D., COSOVIC, B. & KOZARAC, Z. 1983. The adsorption and interaction of long-chain fatty acids and heavy metals at the mercury electrode/sodium chloride solution interface. *Marine Chemistry*, **14**, 17-29.

KUBIAK, W.W. & WANG, J. 1989a. Anodic stripping voltammetry of heavy metals in the presence of organic surfactants. *Talanta*, **36**, 821-824.

KUBIAK, W.W. & WANG, J. 1989b. Use of silica for adsorptive stripping voltammetry in the presence of organic surfactants. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **258**, 41-48.

KUBIAK, W.W. & KOWALSKI, Z. 1989. Use of fumed silica to remove surfactant interferences in continuous flow polarographic measurements. *Analytical Chemistry*, **61**, 1598-1600.

KUKKONEN, J. 1989. Effects of pH and natural humic substances on the accumulation of organic pollutants into freshwater invertebrates. In: *Proceedings of the International Symposium on Humic Substances in the Aquatic and Terrestrial Environment*, Linkoping, Sweden.

KUKKONEN, J., OIKARI, A., JOHNSEN, S. & GJESSING, E. 1989. Effects of humus concentrations on benzo(a)pyrene accumulation from water to *Daphnia magna*: comparison of natural waters and standard preparations. *The Science of the Total Environment*, **79**, 197-207.

KYLE, J.H. 1987. The variation in apparent trace metal complexing capacity of natural waters with plating potential using anodic stripping voltammetry. *Environmental Technology Letters*, **8**, 181-188.

LADD, J.N. & BRISBANE, P.G. 1967. Release of amino acids from soil humic acids by proteolytic enzymes. *Australian Journal of Soil Research*, **5**, 161-171.

LaFRANCE, P., BANTON, O., CAMPBELL, P.G.C. & VILLENEUVE, J-P. 1989. Modeling solute transport in soils in the presence of dissolved humic substances. *The Science of the Total Environment*, **86**, 207-221.

LAMY, I., CROMER, M. & SCHARFF, J.P. 1988. Comparative study of copper(II) interactions with monomeric ligands and synthetic or natural organic materials from potentiometric data. *Analytica Chimica Acta*, **212**, 105-122.

LANDRUM, P.F., NIHART, S.R., EADIE, B.J. & GARDNER, W.S. 1984. Reverse-phase separation method for determining pollutant binding to Aldrich humic acid and dissolved organic carbon in natural waters. *Environmental Science and Technology*, **18**, 187-192.

LANDRUM, P.F. 1989. Bioavailability and toxicokinetics of polycyclic aromatic hydrocarbons sorbed to sediments for the amphipod *Pontoporeia hoyi*. *Environmental Science and Technology*, **23**, 588-595.

LAURENT, T.C. & KILLANDER, J. 1964. A theory of gel filtration and its experimental verification. *Journal of Chromatography*, **14**, 317-330.

LAVIGNE, J.A., LANGFORD, C.H. & MAK, M.K.S. 1987. Kinetic study of speciation of nickel(II) bound to a fulvic acid. *Analytical Chemistry*, **59**, 2616-2620.

LAW, I.A., HAYES, M.H.B. & TUCK, J.J. 1984. Extraction of humic substances from soil using acidified dimethyl sulphoxide. In: *Volunteered Papers 2nd International Conference, International Humic Substances Society*, M.H.B. Hayes, R.S. Swift & J.J. Tuck (eds), University of Birmingham Printing Section, pp 18-21.

LEENHEER, J.A. & HUFFMAN, E.W.D. Jr. 1976. Classification of organic solutes in water by using macroreticular resins. *Journal of Research. US Geological Survey*, **4**, 737-751.

LEENHEER, J.A. 1981. Comprehensive approach to preparative isolation and fractionation of dissolved organic carbon from natural waters and wastewaters. *Environmental Science and Technology*, **15**, 578-587.

LEENHEER, J.A. 1984. Concentration, partitioning, and isolation techniques. In: *Water Analysis Vol (III). Organic Species*. R.A. Minear & L.H. Keith (eds), Academic Press, London, pp 83-166.

LEENHEER, J.A. & NOYES, T.I. 1984. A filtration and column-adsorption system for onsite concentration and fractionation of organic substances from large volumes of water. *US Geological Survey Water-Supply Paper*, **2230**, 16 pp.

LEENHEER, J.A. 1985. Fractionation techniques for aquatic humic substances. In: *Humic Substances in Soil, Sediment, and Water: Geochemistry, Isolations, and Characterization*. G.R. Aiken, D.M. McKnight, R.L. Wershaw & P. MacCarthy (eds), Wiley-Interscience, New York, pp 409-429.

LEENHEER, J.A., WILSON, M.A. & MALCOLM, R.L. 1987. Presence and potential significance of aromatic-ketone groups in aquatic humic substances. *Organic Geochemistry*, **11**, 273-280.

LEENHEER, J.A., BROWN, P.A. & NOYES, T.I. 1989a. Implications of mixture characteristics on humic-substance chemistry. In: *Aquatic Humic Substances. Influence on Fate and Treatment of Pollutants*. I.H. Suffet & P. MacCarthy (eds), Advances in Chemistry Series, Vol 219, American Chemical Society, Washington DC, pp 25-39.

LEENHEER, J.A., McKNIGHT, D.M., THURMAN, E.M. & MacCARTHY, P. 1989b. Structural components and proposed structural models of fulvic acid from the Suwannee River. In: *Humic Substances in the Suwannee River, Georgia: Interactions, Properties, and Proposed Structures*. R.C. Averett, J.A. Leenheer, D.M. McKnight & Thorn, K.A. (eds), U.S. Geological Survey Open-File Report **87-557**, pp 331-359.

LEENHEER, J.A., WERSHAW, R.L. & REDDY, M.M. Structural studies of fulvic acid from the Suwannee River, Georgia: detection, quantification, and significance of substituted malonic-acid groups. (in preparation).

LEENHEER, J.A. & NOYES, T.I. Derivatization of humic substances for structural studies. In: *Humic Substances in Soil, Sediment, and Water II. Structures and Interactions*. M.H.B. Hayes, P. MacCarthy, R.L. Malcolm & R.S. Swift (eds), Wiley-Interscience (in press).

LEGGETT, D.J. (ed). 1985. *Computational Methods for the Determination of Formation Constants*. Plenum Press, New York.

L'EPLATTENIER, F., MURASE, I. & MARTEL, A.E. 1967. New multidentate ligands. VI. Chelating tendencies of N,N'-di(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic acid. *Journal of the American Chemical Society*, **89**, 837-843.

LEPPARD, G.G., BUFFLE, J. & BAUDAT, R. 1986. A description of the aggregation properties of aquatic pedogenic fulvic acids. Combining physico-chemical data and microscopical observations. *Water Research*, **20**, 185-196.

LETTERMAN, R.D. & ASOLEKAR, S.R. 1990a. Surface ionization of polynuclear species in Al(III) hydrolysis -I. Titration results. *Water Research*, **24**, 931-939.

LETTERMAN, R.D. & ASOLEKAR, S.R. 1990b. Surface ionization of polynuclear species in Al(III) hydrolysis -II. A conditional equilibrium model. *Water Research*, **24**, 941-948.

LEUENBERGER, B. & SCHINDLER, P.W. 1986. Application of integral pK spectrometry to the titration curve of fulvic acid. *Analytical Chemistry*, **58**, 1471-1474.

LEUNG, V.W.-H., DARVELL, B.W. & CHAN, A.P.-C. 1988. A rapid algorithm for solution of the equations of multiple equilibrium systems - RAMESES. *Talanta*, **35**, 713-718.

LEVERSEE, G.J., LANDRUM, P.F., GIESY, J.P. & FANNIN, T. 1983. Humic acids reduce bioaccumulation of some polycyclic aromatic hydrocarbons. *Canadian Journal of Fisheries and Aquatic Sciences*, **40**, 63-69.

LEWIN, J.C. 1962. Silicification. In: *Physiology and Biochemistry of Algae*, R.A. Lewin (ed), Academic Press, New York, pp 445-455.

LIM, M.C. 1978. Mixed-ligand complexes of palladium(II). Part 3. Diaqua(ethylenediamine)palladium(II) complexes of L-amino acids. *Journal of the Chemical Society - Dalton Transactions*, 726-728.

LOBARTINI, J.C., TAN, K.H., ASMUSSEN, L.E., LEONARD, R.A., HIMMELSBACH, D. & GINGLE, A.R. 1989. Humic matter isolated from soils and water by the XAD-8 resin and conventional NaOH methods. *Communications in Soil Science and Plant Analysis*, **20**, 1453-1477.

LOCHMÜLLER, C.H. & SAAVEDRA, S.S. 1986. Conformational changes in a soil fulvic acid measured by time-dependent fluorescence depolarization. *Analytical Chemistry*, **58**, 1978-1981.

LOMOZIK, L. 1984. A study of complex equilibria of phenylglycine with nickel(II), copper(II) and zinc(II) in water and in water-methanol solution. *Monatshefte für Chemie*, **115**, 921-926.

LOMOZIK, L. & WOJCIECHOWSKA, A. 1985. Complexing properties of methyl and phenyl glycine derivatives in their compounds with H^+, Ni(II), Cu(II), and Zn(II). *Monatshefte für Chemie*, **116**, 719-727.

LUKASZEWSKI, Z., PAWLAK, M.K. & CISZEWSKI, A. 1979. Determination of the stage of the process deciding of the total effect of the influence of organic substances on the peaks in anodic stripping voltammetry. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **103**, 217-223.

LUMSDEN, B.R. & FLORENCE, T.M. 1983. A new algal assay procedure for the determination of the toxicity of copper species in seawater. *Environmental Technology Letters*, **4**, 271-276.

LUND, W. 1986. Electrochemical methods and their limitations for the determination of metal species in natural waters. In: *The Importance of Chemical "Speciation" in Environmental Processes*. M. Bernhard, F.E. Brinckman & P.J. Sadler (eds), Springer-Verlag, Berlin, pp 533-561.

LYTLE, C.R. & PERDUE, E.M. 1981. Free, proteinaceous, and humic-bound amino acids in river water containing high concentrations of aquatic humus. *Environmental Science and Technology*, **15**, 224-228.

MacCARTHY, P. 1976. A proposal to establish a reference collection of humic materials for interlaboratory comparisons. *Geoderma*, **16**, 179-181.

MacCARTHY, P., PETERSON, M.J., MALCOLM, R.L. & THURMAN, E.M. 1979. Separation of humic substances by pH gradient desorption from a hydrophobic resin. *Analytical Chemistry*, **51**, 2041-2043.

MacCARTHY, P. & RICE, J.A. 1985. Spectroscopic methods (other than NMR) for determining functionality in humic substances. In: *Humic Substances in Soil, Sediment, and Water. Geochemistry, Isolation, and Characterization.* G.R. Aiken, D.M. McKnight, R.L. Wershaw & P. MacCarthy (eds), Wiley-Interscience, New York, pp 527-559.

MacCARTHY, P., DE LUCA, S.J., VOORHEES, K.J., MALCOLM, R.L. & THURMAN, E.M. 1985. Pyrolysis-mas spectrometry/pattern recognition on a well-characterized suite of humic samples. *Geochimica et Cosmochimica Acta*, **49**, 2091-2096.

MacCARTHY, P. & MALCOLM, R.L. 1989. The nature of commercial humic acids. In: *Aquatic Humic Substances. Influence on Fate and Treatment of Pollutants.* I.H. Suffet & P. MacCarthy (eds), Advances in Chemistry Series Vol 219, American Chemical Society, Washington, pp 55-63.

MacCARTHY, P. & SUFFET, I.H. 1989. Aquatic humic substances and their influence on the fate and treatment of pollutants. In: *Aquatic Humic Substances. Influence on Fate and Treatment of Pollutants.* I.H. Suffet & P. MacCarthy (eds), Advances in Chemistry Series, Vol 219, American Chemical Society, Washington DC, pp xvii-xxx.

MacINTYRE, W.G., SMITH, C.L., CHIOU, C.T., PORTER, P.E. & SHOUP, T.D. 1984. Comment on "Partition equilibria of nonionic compounds between soil organic matter and water". *Environmental Science and Technology*, **18**, 295-297.

MACKEY, D.J. 1982a. The adsorption of simple trace metal cations on XAD-1 and XAD-2. A study using a multichannel non-dispersive atomic fluorescence detector with quantitation by batch measurements. *Journal of Chromatography*, **236**, 81-95.

MACKEY, D.J. 1982b. Cation-exchange behaviour of a range of adsorbents and chromatographic supports with regard to their suitability for investigating trace metal speciation in natural waters. *Journal of Chromatography*, **242**, 275-287.

MACKEY, D.J. 1983. Metal-organic complexes in seawater - an investigation of naturally occurring complexes of Cu, Zn, Fe, Mg, Ni, Cr, Mn and Cd using high-performance liquid chromatography with atomic fluorescence detection. *Marine Chemistry*, **13**, 169-180.

MAGGI, L., STELLA, R. & CICERI, G. 1984. Isolation and characterization of organic matter from river water: copper(II) and cadmium(II) interactions with the fulvic component. *Annali di Chimica*, **74**, 257-267.

MAK, M.K.S. & LANGFORD, C.H. 1982. A kinetic study of the interaction of hydrous aluminum oxide colloids with a well-characterized soil fulvic acid. *Canadian Journal of Chemistry*, **60**, 2023-2028.

MALCOLM, R.L. 1976. Method and importance of obtaining humic and fulvic acids of high purity. *Journal of Research. U.S. Geological Survey*, **4**, 37-40.

MALCOLM, R.L. 1985. Geochemistry of stream fulvic and humic substances. In: *Humic Substances in Soil, Sediment, and Water. Geochemistry, Isolation, and Characterization.* G.R. Aiken, D.M. McKnight, R.L. Wershaw & P. MacCarthy (eds), Wiley-Interscience, New York, pp 181-209.

MALCOLM, R.L. & MacCARTHY, P. 1986. Limitations in the use of commercial humic acids in water and soil research. *Environmental Science and Technology*, **20**, 904-911.

MALCOLM, R.L. 1990. The uniqueness of humic substances in each of soil, stream and marine environments. *Analytica Chimica Acta*, **232**, 19-30.

MANN, K.J. & FLORENCE, T.M. 1987a. Anodic stripping voltammetry with medium exchange in trace element speciation. *Analytica Chimica Acta*, **200**, 305-312.

MANN, K.J. & FLORENCE, T.M. 1987b. Trace element speciation by anodic stripping voltammetry: the effects of added mercuric and acetate ions. *The Science of the Total Environment*, **60**, 67-74.

MANTOURA, R.F.C. & RILEY, J.P. 1975a. The analytical concentration of humic substances from natural waters. *Analytica Chimica Acta*, **76**, 97-106.

MANTOURA, R.F.C. & RILEY, J.P. 1975b. The use of gel filtration in the study of metal binding by humic acids and related compounds. *Analytica Chimica Acta*, **78**, 193-200.

MANTOURA, R.F.C., DICKSON, A. & RILEY, J.P. 1978. The complexation of metals with humic materials in natural waters. *Estuarine, Coastal and Marine Science*, **6**, 387-408.

MANTOURA, R.F.C. & WOODWARD, E.M.S. 1983. Conservative behaviour of riverine dissolved organic carbon in the Severn Estuary, chemical and geochemical implications. *Geochimica et Cosmochimica Acta*, **47**, 1293-1309.

MARATHE, D.G., AMBULKAR, R.S. & MUNSHI, K.N. 1984. Physico-chemical investigation on the complexes of gallium(III) with hydroxy amino substituted carboxylic acids and hydroxy ketones. *National Academy of Science Letters*, **7**, 153-156.

MARINSKY, J.A., GUPTA, S. & SCHINDLER, P. 1982a. The interaction of Cu(II) ion with humic acid. *Journal of Colloid and Interface Science*, **89**, 401-411.

MARINSKY, J.A., GUPTA, S. & SCHINDLER. P. 1982b. A unified physicochemical description of the equilibria encountered in humic acid gels. *Journal of Colloid and Interface Science*, **89**, 412-426.

MARINSKY, J.A. & EPHRAIM, J. 1986. A unified physicochemical description of the protonation and metal ion complexation equilibria of natural organic acids (humic and fulvic acids). 1. Analysis of the influence of polyelectrolyte properties on protonation equilibria in ionic media: fundamental concepts. *Environmental Science and Technology*, **20**, 349-354.

MARINSKY, J.A. & REDDY, M.M. 1990. Vapor-pressure osmometric study of the molecular weight and aggregation tendency of a reference-soil fulvic acid. *Analytica Chimica Acta*, **232**, 123-130.

MARKLUND, E., SJÖBERG, S. & ÖHMAN, L.-O. 1986. Equilibrium and structural studies of silicon(IV) and aluminium(III) in aqueous solution. 14. Speciation and equilibria in the aluminium(III)-lactic acid-OH^- system. *Acta Chemica Scandinavica*, **A40**, 367-373.

MARKLUND, E. & ÖHMAN, L-O. 1990. Equilibrium and structural studies of silicon(IV) and aluminium(III) in aqueous solution. 25. Composition and stability of aluminium complexes with methylmalonic acid and alanine. *Acta Chemica Scandinavica*, **44**, 353-357.

MARLEY, N.A., BENNETT, P., JANECKY, D.R. & GAFFNEY, J.S. 1989. Spectroscopic evidence for organic diacid complexation with dissolved silica in aqueous systems - I. Oxalic acid. *Organic Geochemistry*, **14**, 525-528.

MART, L., NURNBERG, H.W. & VALENTA, P. 1980. Prevention of contamination and other accuracy risks in voltammetric trace metal analysis of natural waters. Part III. Voltammetric ultratrace analysis with a multicell system designed for clean bench working. *Fresenius Zeitschrift für Analytische Chemie*, **300**, 350-362.

MARTIN, C.R. & DOLLARD, K.A. 1983. Effect of hydrophobic interactions on the rates of ionic diffusion in Nafion films at electrode surfaces. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **159**, 127-135.

MARTY, J.C., ZUTIC, V., PRECALI, R., SALIOT, A., COSOVIC, B., SMODLAKA, N. & CAUWET, G. 1988. Organic matter characterization in the northern Adriatic Sea with special reference to the sea surface microlayer. *Marine Chemistry*, **25**, 243-263.

MATHUR, S.P. & PAUL, E.A. 1966. A microbiological approach to the problem of soil humic acid structures. *Nature*, **212**, 646-647.

MATHUR, S.P. & PAUL, E.A. 1967a. Microbial utilization of soil humic acids. *Canadian Journal of Microbiology*, **13**, 573-580.

MATHUR, S.P. & PAUL, E.A. 1967b. Partial characterization of soil humic acids through biodegradation. *Canadian Journal of Microbiology*, **13**, 581-586.

MATHUR, S.P. 1971. Characterization of soil humus through enzymatic degradation. *Soil Science*, **111**, 147-157.

MATTHEWS, R.W., ABDULLAH, M. & LOW, G.K.-C. 1990. Photocatalytic oxidation for total organic carbon analysis. *Analytica Chimica Acta*, **233**, 171-179.

MATTUSCH, J., HALLMEIER, K-H., STULIK, K. & PACAKOVA, V. 1989. Pretreatment of glassy carbon electrodes by anodic galvanostatic pulses with a large amplitude. *Electroanalysis*, **1**, 405-412.

MAY, P.M., WILLIAMS, D.R., LINDER, P.W. & TORRINGTON, R.G. 1982. The use of glass electrodes for the determination of formation constants - I. A definitive method for calibration. *Talanta*, **29**, 249-256.

MAY, P.M. & WILLIAMS, D.R. 1985. MAGEC: A program for the definitive calibration of the glass electrode. In: *Computational Methods for the Determination of Formation Constants*. D.J. Leggett (ed), Plenum Press, New York, pp 37-70.

MAY, P.M., MURRAY, K. & WILLIAMS, D.R. 1985. The use of glass electrodes for the determination of formation constants - II. Simulation of titration data. *Talanta*, **32**, 483-489.

MAY, P.M. & MURRAY, K. 1988. The use of glass electrodes for the determination of formation constants - IV. Matters of weight. *Talanta*, **35**, 927-932.

MAY, P.M., MURRAY, K. & WILLIAMS, D.R. 1988. The use of glass electrodes for the determination of formation constants - III. Optimization of titration data: the ESTA library of computer programs. *Talanta*, **35**, 825-830.

MAY, P.M. & MURRAY, K. The use of glass electrodes for the determination of formation constants - VI. Recommended procedures for publication. (in press).

MAYER, L.M. 1985. Geochemistry of humic substances in estuarine environments. In: *Humic Substances in Soil, Sediment, and Water. Geochemistry, Isolation, and Characterization*. G.R. Aiken, D.M. McKnight, R.L. Wershaw & P. MacCarthy (eds), Wiley-Interscience, New York, pp 211-232.

McBRYDE, W.A.E. 1968. Constraints on the determination of stability constants for metal complexes. II. The iron(III) phenolates. *Canadian Journal of Chemistry*, **46**, 2385-2392.

McBRYDE, W.A.E. 1969. The pH meter as a hydrogen-ion concentration probe. *The Analyst*, **94**, 337-346.

McCARTHY, J.F.1983. Role of particulate organic matter in decreasing accumulation of polynuclear aromatic hydrocarbons by *Daphnia magna*. *Archives of Environmental Contamination and Toxicology*, **12**, 559-568.

McCARTHY, J.F. & JIMENEZ, B.D. 1985a. Interactions between polycyclic aromatic hydrocarbons and dissolved humic material: binding and dissociation. *Environmental Science and Technology*, **19**, 1072-1076.

McCARTHY, J.F. & JIMENEZ, B.D. 1985b. Reduction in bioavailability to bluegills of polycyclic aromatic hydrocarbons bound to dissolved humic material. *Environmental Toxicology and Chemistry*, **4**, 511-521.

McCARTHY, J.F., JIMENEZ, B.D. & BARBEE, T. 1985. Effect of dissolved humic material on accumulation of polycyclic aromatic hydrocarbons: structure-activity relationships. *Aquatic Toxicology*, **7**, 15-24.

McCARTHY, J.F. 1989. Bioavailability and toxicity of metals and hydrophobic organic contaminants. In: *Aquatic Humic Substances. Influence on Fate and Treatment of Pollutants*, I.H. Suffet & P. MacCarthy (eds), American Chemical Society, Advances in Chemistry Series 219, Washington DC, pp 263-277.

McCARTHY, J.F. & ZACHARA, J.M. 1989. Subsurface transport of contaminants. *Environmental Science and Technology*, **23**, 496-502.

McCARTHY, J.F., ROBERSON, L.E. & BURRUS, L.W. 1989. Association of benzo(a)pyrene with dissolved organic matter: prediction of K_{dom} from structural and chemical properties of the organic matter. *Chemosphere*, **19**, 1911-1920.

McKEAGUE, J.A. 1967. An evaluation of 0.1 M pyrophosphate and pyrophosphate-dithionite in comparison with oxalate as extractants of the accumulation products in Podzols and some other soils. *Canadian Journal of Soil Science*, **47**, 95-99.

McKNIGHT, D.M. & MOREL, F.M.M. 1979. Release of weak and strong copper-complexing agents by algae. *Limnology and Oceanography*, **24**, 823-837.

McKNIGHT, D.M. & WERSHAW, R.L. 1989. Complexation of copper by fulvic acid from the Suwannee River. Effect of counter-ion concentration. In: *Humic Substances in the Suwannee River, Georgia: Interactions, Properties, and Proposed Structures*. R.C. Averett, J.A. Leenheer, D.M. McKnight & K.A. Thorn (eds), U.S. Geological Survey Open-File Report, **87-557**, pp 59-79.

MEANS, J.C. & WIJAYARATNE, R. 1982. Role of natural colloids in the transport of hydrophobic pollutants. *Science*, **215**, 968-970.

MEHLHORN, R.J. 1986. The interaction of inorganic species with biomembranes. In: *The Importance of Chemical "Speciation" in Environmental Processes*. M. Bernhard, F.E. Brinckman & P.J. Sadler (eds), Springer-Verlag, Berlin, pp 85-97.

MELOUN, M., BARTOS, M. & HOGFELDT, E. 1988. Multiparametric curve fitting - XIII. Reliability of formation constants determined by analysis of potentiometric titration data. *Talanta*, **35**, 981-991.

MIANO, T.M., SPOSITO, G. & MARTIN, J.P. 1988. Fluorescence spectroscopy of humic substances. *Soil Science Society of America Journal*, **52**, 1016-1019.

MIDORIKAWA, T., TANOUE, E. & SUGIMURA, Y. 1990. Determination of complexing ability of natural ligands in seawater for various metal ions using ion selective electrodes. *Analytical Chemistry*, **62**, 1737-1746.

MIKITA, M.A., STEELINK, C. & WERSHAW, R.L. 1981. Carbon-13 enriched nuclear magnetic resonance method for the determination of hydroxyl functionality in humic substances. *Analytical Chemistry*, **53**, 1715-1717.

MILLINGTON, L.A., GOULDING, K.H. & ADAMS, N. 1988. The influence of growth medium composition on the toxicity of chemicals to algae. *Water Research*, **22**, 1593-1597.

MILLS, G.L. & QUINN, J.G. 1981. Isolation of dissolved organic matter and copper-organic complexes from estuarine waters using reverse-phase liquid chromatography. *Marine Chemistry*, **10**, 93-102.

MILLS, G.L., HANSON, A.K. Jr., QUINN, J.G., LAMMELA, W.R. & CHASTEEN, N.D. 1982. Chemical studies of copper-organic complexes isolated from estuarine waters using C_{18} reverse-phase liquid chromatography. *Marine Chemistry*, **11**, 355-377.

MILLS, G.L. & QUINN, J.G. 1984. Dissolved copper and copper-organic complexes in the Narragansett Bay estuary. *Marine Chemistry*, **15**, 151-172.

MILLS, G.L., McFADDEN, E. & QUINN, J.G. 1987. Chromatographic studies of dissolved organic matter and copper-organic complexes isolated from estuarine waters. *Marine Chemistry*, **20**, 313-325.

MINDERMAN, G. 1979a. A tentative approach to the molecular structure of humic acids: the spectral evidence for a derivation of humic acids from plant-borne esters. 1. The electron paramagnetic resonance (EPR) spectra. *Netherlands Journal of Agricultural Science*, **27**, 79-91.

MINDERMAN, G. 1979b. A tentative approach to the molecular structure of humic acids: the spectral evidence for a derivation of humic acids from plant-borne esters. 2. Infrared and chemical analyses. *Netherlands Journal of Agricultural Science*, **27**, 153-175.

MINDERMAN, G. 1979c. A tentative approach to the molecular structure of humic acids: the spectral evidence for a derivation of humic acids from plant-borne esters. 3. Pyrolysis-mass spectrometry. *Netherlands Journal of Agricultural Science*, **27**, 277-283.

MINGELGRIN, U. & GERSTL, Z. 1983. Reevaluation of partitioning as a mechanism of nonionic chemicals adsorption in soils. *Journal of Environmental Quality*, **12**, 1-11.

MORI, S., HIRAIDE, M. & MIZUIKE, A. 1987. Aqueous size-exclusion chromatography of humic acids on a Sephadex gel column with diluted phosphate buffers as eluents. *Analytica Chimica Acta*, **193**, 231-238.

MORRISON, G.M.P. 1989. Trace metal speciation and its relationship to bioavailability and toxicity in natural waters. In: *Trace Element Speciation: Analytical Methods and Problems*, G.E. Batley (ed), CRC Press Inc., Florida, pp 25-41.

MORRISON, G.M.P. & FLORENCE, T.M. 1989a. Comparison of physicochemical speciation procedures with metal toxicity to *Chlorella pyrenoidosa*. Copper complexation capacity. *Electroanalysis*, **1**, 107-112.

MORRISON, G.M.P. & FLORENCE, T.M. 1989b. Electrochemical speciation analysis of metals at membrane-coated electrodes. *Electroanalysis*, **1**, 485-491.

MORRISON, G.M.P., FLORENCE, T.M. & STAUBER, J.L. 1990. The effects of complexing agents and surfactants on the deposition and stripping processes in differential pulse anodic stripping voltammetry of metals at the hanging mercury drop electrode. *Electroanalysis*, **2**, 9-14.

MOTA, A.M., BUFFLE, J., KOUNAVES, S.P. & GONCALVES, M.L.S. 1985. The importance of concentration effects at the electrode surface in anodic stripping voltammetric measurements of complexation of metal ions at natural water concentrations. *Analytica Chimica Acta*, **172**, 13-30.

MULDER, J., VAN BREEMEN, N. & EIJCK, H.C. 1989. Depletion of soil aluminium by acid deposition and implications for acid neutralization. *Nature*, **337**, 247-249.

MURPHY, E.M., ZACHARA, J.M. & SMITH, S.C. 1990. Influence of mineral-bound humic substances on the sorption of hydrophobic organic compounds. *Environmental Science and Technology*, **24**, 1507-1516.

MURRAY, K. & LINDER, P.W. 1983. Fulvic acids: structure and metal binding. I. A random molecular model. *Journal of Soil Science*, **34**, 511-523.

NAKAGAWA, G., WADA, H. & SAKO, T. 1980. Performances of lead and copper(II) ion-selective electrodes in metal buffer solutions and the determination of the stability constants of lead and copper(II) complexes. *Bulletin. Chemical Society of Japan*, **53**, 1303-1307.

NANCOLLAS, G.H. & TOMSON, M.B. 1982. Guidelines for the determination of stability constants. *Pure and Applied Chemistry*, **54**, 2675-2692.

NEBEKER, A.V., SCHUYTEMA, G.S., GRIFFIS, W.L., BARBITTA, J.A. & CAREY, L.A. 1989. Effect of sediment organic carbon on survival of *Hyalella azteca* exposed to DDT and endrin. *Environmental Toxicology and Chemistry*, **8**, 705-718.

NELSON, A. & MANTOURA, R.F.C. 1984. Voltammetry of copper species in estuarine waters. Part III. Use of gelatin to investigate adsorption of natural organic material on the HMDE during DPASV. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **164**, 265-272.

NELSON, A. 1985a. Voltammetry of copper species in estuarine waters. Induced adsorption of copper on the hanging mercury drop electrode in complexing ligand/surfactant/chloride media. *Analytica Chimica Acta*, **169**, 273-286.

NELSON, A. 1985b. Voltammetric measurement of copper(II)/organic interactions in estuarine waters. *Analytica Chimica Acta*, **169**, 287-298.

NELSON, A. & DONKIN, P. 1985. Processes of bioaccumulation: the importance of chemical speciation. *Marine Pollution Bulletin*, **16**, 164-169.

NELSON, A., AUFFRET, N. & READMAN, J. 1988. Initial applications of phospholipid-coated mercury electrodes to the determination of polynuclear aromatic hydrocarbons and other organic micropollutants in aqueous systems. *Analytica Chimica Acta*, **207**, 47-57.

NEUBECKER, T.A. & ALLEN, H.E. 1983. The measurement of complexation capacity and conditional stability constants for ligands in natural waters. *Water Research*, **17**, 1-14.

NEYROUD, J.A. & SCHNITZER, M. 1975. The alkaline hydrolysis of humic substances. *Geoderma*, **13**, 171-188.

NIP, M., TEGELAAR, E.W., DE LEEUW, J.W. & SCHENCK, P.A. 1986. A new non-saponifiable highly aliphatic and resistant biopolymer in plant cuticles. Evidence from pyrolysis and ^{13}C-NMR analysis of present-day and fossil plants. *Naturwissenschaften*, **73**, 579-585.

NIP, M., DE LEEUW, J.W., HOLLOWAY, P.J., JENSEN, J.P.T., SPRENKELS, J.C.M., DE POOTER, M. & SLEECKX, J.J.M. 1987. Comparison of flash pyrolysis, differential scanning calorimetry, ^{13}C NMR and IR spectroscopy in the analysis of a highly aliphatic biopolymer from plant cuticles. *Journal of Analytical and Applied Pyrolysis*, **11**, 287-295.

NISSENBAUM, A. & KAPLAN, I.R. 1972. Chemical and isotopic evidence for the in situ origin of marine humic substances. *Limnology and Oceanography*, **17**, 570-582.

NISSENBAUM, A. & SWAINE, D.J. 1976. Organic matter-metal interactions in Recent sediments: the role of humic substances. *Geochimica et Cosmochimica Acta*, **40**, 809-816.

NURNBERG, H.W. 1983. Investigations on heavy metal speciation in natural waters by voltammetric procedures. *Fresenius Zeitschrift für Analytische Chemie*, **316**, 557-565.

OGNER, G. & SCHNITZER, M. 1971. Chemistry of fulvic acid, a soil humic fraction, and its relation to lignin. *Canadian Journal of Chemistry*, **49**, 1053-1063.

OGURA, N. 1974. Molecular weight fractionation of dissolved organic matter in coastal seawater by ultrafiltration. *Marine Biology*, **24**, 305-312.

ÖHMAN, L-O. & FORSLING, W. 1981. Equilibrium and structural studies of silicon(IV) and aluminium(III) in aqueous solution. 3. A potentiometric study of aluminium(III) hydrolysis and aluminium(III) hydroxo carbonates in 0.6 M Na(Cl). *Acta Chemica Scandinavica*, **A35**, 795-802.

ÖHMAN, L-O. & SJÖBERG, S. 1983. Equilibrium and structural studies of silicon(IV) and aluminium(III) in aqueous solution. Part 9. A potentiometric study of mono- and poly-nuclear aluminium(III) citrates. *Journal of the Chemical Society - Dalton Transactions*, 2513-2517.

ÖHMAN, L-O. 1988. Equilibrium and structural studies of silicon(IV) and aluminium(III) in aqueous solution. 17. Stable and metastable complexes in the system H^+-Al^{3+}-citric acid. *Inorganic Chemistry*, **27**, 2565-2570.

OLSON, D.L. & SHUMAN, M.S. 1985. Copper dissociation from estuarine humic materials. *Geochimica et Cosmochimica Acta*, **49**, 1371-1375.

OPPERMAN, J.H., VAN STADEN, J.F. & BOHMER, R.G. 1988. Effects of certain surfactants on the voltammetric determination of copper, lead, and cadmium. *South African Journal of Chemistry*, **41**, 26-32.

OPPERHUIZEN, A., VAN DER WELDE, E.W., GOBAS, F.A.P.C., LIEM, D.A.K. & VAN DER STEEN, J.M.D. 1985. Relationship between bioconcentration in fish and steric factors of hydrophobic chemicals. *Chemosphere*, **14**, 1871-1896.

OREM, W.H. & HATCHER, P.G. 1987. Solid-state ^{13}C NMR studies of dissolved organic matter in pore waters from different depositional environments. *Organic Geochemistry*, **11**, 73-82.

ORIS, J.T., HALL, A.T. & TYLKA, J.D. 1990. Humic acids reduce the photo-induced toxicity of anthracene to fish and daphnia. *Environmental Toxicology and Chemistry*, **9**, 575-583.

ORLOV, D.S., AMMOSOVA, Y.M., GLEBOVA, G.I., GORSHKOVA, Y.I., IL'IN, N.P. & KOLESNIKOV, M.P. 1971. Molecular weight, sizes, and configuration of humic-acid particles. *Soviet Soil Science*, **3**, 673-687.

ORLOV, D.S., PIVOVAROVA, I.A. & GORBUNOV, N.I. 1973. Interaction of humic substances with minerals and the nature of their bond - a review. *Soviet Soil Science*, **5**, 568-581.

ORLOV, D.S., AMMOSOVA, Y.M. & GLEBOVA, G.I. 1975. Molecular parameters of humic acids. *Geoderma*, **13**, 211-229.

ORLOV, D.S., MIN'KO, O.I., DEMIN, V.V., SAL'NIKOV, V.G., IZMAYLOVA, N.B. & MILANOVSKIY, Y.Y. 1990. Involvement of metals in the molecular weight organization of humic substances. *Soviet Soil Science*, **22**, 70-73.

O'SHEA, T.A. & MANCY, K.H. 1978. The effect of pH and hardness metal ions on the competitive interaction between trace metal ions and inorganic and organic complexing agents found in natural waters. *Water Research*, **12**, 703-711.

PAGÉ, F. & DE KIMPE, C.R. 1989. Dissolution des composés ferrugineux et alumineux des horizons B Podzoliques de sols du Québec par le dithionite-citrate-bicarbonate, l'oxalate, le pyrophosphate et le tétraborate. *Canadian Journal of Soil Science*, **69**, 451-459.

PALEOS, J. 1969. Adsorption from aqueous and nonaqueous solutions on hydrophobic and hydrophilic high surface-area copolymers. *Journal of Colloid and Interface Science*, **31**, 7-18.

PATIENCE, R.L. & WILSON, M.A. 1990. Practical application of solid state ^{13}C NMR to the structural elucidation of sedimentary organic matter. *Trends in Analytical Chemistry*, **9**, 26-31.

PAXÉUS, N. & WEDBORG, M. 1985. Acid-base properties of aquatic fulvic acid. *Analytica Chimica Acta*, **169**, 87-98.

PEASE, B.F. & WILLIAMS, M.B. 1959. Spectrophotometric investigation of the analytical reagent 1-(2-pyridylazo)-2-naphthol and its copper chelate. *Analytical Chemistry*, **31**, 1044-1047.

PERDUE, E.M. 1978. Solution thermochemistry of humic substances -I. Acid-base equilibria of humic acid. *Geochimica et Cosmochimica Acta*, **42**, 1351-1358.

PERDUE, E.M. & WOLFE, N.L. 1982. Modification of pollutant hydrolysis kinetics in the presence of humic substances. *Environmental Science and Technology*, **16**, 847-852.

PERDUE, E.M. 1983. Association of organic pollutants with humic substances: partitioning equilibria and hydrolysis kinetics. *In: Aquatic and Terrestrial Humic Materials*, R.F. Christman & E.T. Gjessing (eds), Ann Arbor Science, Ann Arbor, Michigan, pp 441-460.

PERDUE, E.M. & LYTLE, C.R. 1983a. A distribution model for binding of protons and metal ions by humic substances. *Environmental Science and Technology*, **17**, 654-660.

PERDUE, E.M. & LYTLE, C.R. 1983b. A critical examination of metal-ligand complexation models: application to defined multiligand mixtures. In: *Aquatic and Terrestrial Humic Materials*. R.F. Christman & E.T. Gjessing (eds), Ann Arbor Science, Ann Arbor, pp 295-313.

PERDUE, E.M. 1984. Analytical constraints on the structural features of humic substances. *Geochimica et Cosmochimica Acta*, **48**, 1435-1442.

PERDUE, E.M., REUTER, J.H. & PARRISH, R.S. 1984. A statistical model of proton binding by humus. *Geochimica et Cosmochimica Acta*, **48**, 1257-1263.

PERDUE, E.M. 1985. Acidic functional groups of humic substances. In: *Humic Substances in Soil, Sediment, and Water. Geochemistry, Isolation, and Characterization.* G.R. Aiken, D.M. McKnight, R.L. Wershaw & P. MacCarthy (eds), Wiley-Interscience, New York, pp 493-526.

PERDUE, E.M. 1989. Effect of humic substances on metal speciation. In: *Aquatic Humic Substances. Influence on Fate and Treatment of Pollutants.* I.H. Suffet & P. MacCarthy (eds), Advances in Chemistry Series Vol. 219, American Chemical Society, Washington DC, pp 281-295.

PERRIN, D.D. 1965. *Dissociation Constants of Organic Bases in Aqueous Solution.* Butterworths, London.

PERRIN, D.D. 1979. *Stability Constants of Metal-Ion Complexes. Part B. Organic Ligands.* IUPAC Chemical Data Series - No. 22, Pergamon, Oxford.

PETERSON, W.M. & WONG, R.V. 1981. Fundamentals of stripping voltammetry. *American Laboratory*, **13**, 116-128.

PETTIT, L.D. 1984. Critical evaluation of equilibrium constants in solution. Part A: Stability constants of metal complexes. Critical survey of formation constants of complexes of histidine, phenylalanine, tyrosine, L-DOPA and tryptophan. *Pure and Applied Chemistry*, **56**, 247-292.

PFEFFER, P.E., GERASIMOWICZ, W.V. & PIOTROWSKI, E.G. 1984. Effect of paramagnetic iron on quantitation in carbon-13 cross polarization magic angle spinning nuclear magnetic resonance spectrometry of heterogeneous environmental matrices. *Analytical Chemistry*, **56**, 734-741.

PICCOLO, A. & STEVENSON, F.J. 1982. Infrared spectra of Cu^{2+}, Pb^{2+}, and Ca^{2+} complexes of soil humic substances. *Geoderma*, **27**, 195-208.

PICCOLO, A. & MIRABELLA, A. 1987. Molecular weight distribution of peat humic substances extracted with different inorganic and organic solutions. *The Science of the Total Environment*, **62**, 39-46.

PICCOLO, A. 1988. Characteristics of soil humic extracts obtained by some organic and inorganic solvents and purified by HCl-HF treatment. *Soil Science*, **146**, 418-426.

PICCOLO, A., RAUSA, R. & CALEMMA, V. 1989. FT-IR spectra of humic substances extracted with dipolar aprotic solvents. *Chemosphere*, **18**, 1927-1933.

PICCOLO, A., CAMPANELLA, L. & PETRONIO, B.M. 1990. Carbon-13 nuclear magnetic resonance spectra of soil humic substances extracted by different mechanisms. *Soil Science Society of America Journal*, **54**, 750-756.

PIERCE, R.H.Jr., OLNEY, C.E. & FELBECK, G.T. Jr. 1971. Pesticide adsorption in soils and sediments. *Environmental Letters*, **1**, 157-172.

PIERCE, R.H. Jr., OLNEY, C.E. & FELBECK, G.T. Jr. 1974. pp'-DDT adsorption to suspended particulate matter in seawater. *Geochimica et Cosmochimica Acta*, **38**, 1061-1073.

PIETRZYK, D.J. & CHU, C-H. 1977. Amberlite XAD copolymers in reversed phase gravity flow and high pressure liquid chromatography. *Analytical Chemistry*, **49**, 757-764.

PIRET, E.L., WHITE, R.G., WALTHER, H.C. Jr., & MADDEN, A.J.Jr. 1960. Some physico-chemical properties of peat humic acids. *Royal Dublin Society Proceedings, Series A*, **1**, 69-79.

PLAVSIC, M. & COSOVIC, B. 1989. The effect of surface active substances on the electrochemical behaviour of copper ions in chloride solutions and in natural waters. *Water Research*, **23**, 1545-1553.

PLECHANOV, N. 1983. Studies of molecular weight distributions of fulvic and humic acids by gel permeation chromatography. Examination of the solute molecular composition using RI, UV, fluorescence and weight measurement as detection techniques. *Organic Geochemistry*, **5**, 143-149.

PÓCSI, I. & FÁBIÁN, I. 1988. Complex equilibria in aqueous solutions of Ti^{3+}-glycine and -malonic acid. *Journal of the Chemical Society - Dalton Transactions*, 2231-2233.

POIRRIER, M.A., BORDELON, B.R. & LASETER, J.L. 1972. Adsorption and concentration of dissolved carbon-14 DDT by coloring colloids in surface waters. *Environmental Science and Technology*, **6**, 1033-1035.

POMMER, A.M. & BREGER, I.A. 1960a. Equivalent weight of humic acid from peat. *Geochimica et Cosmochimica Acta*, **20**, 45-50.

POMMER, A.M. & BREGER, I.A. 1960b. Potentiometric titration and equivalent weight of humic acid. *Geochimica et Cosmochimica Acta*, **20**, 30-44.

POON, M. & McCREERY, R.L. 1987. Repetitive in situ renewal and activation of carbon and platinum electrodes: applications to pulse voltammetry. *Analytical Chemistry*, **59**, 1615-1620.

POSNER, A.M. 1966. The humic acids extracted by various reagents from a soil. Part I. Yield, inorganic components and titration curves. *Journal of Soil Science*, **17**, 65-78.

POTT, D.B., ALBERTS, J.J. & ELZERMAN, A.W. 1985. The influence of pH on the binding capacity and conditional stability constants of aluminum and naturally-occurring organic matter. *Chemical Geology*, **48**, 293-304.

POWELL, H.K.J. & TAYLOR, M.C. 1983. A comment on the simultaneous determination of glass electrode parameters and protonation constants. *Talanta*, **30**, 885-886.

POWELL, H.K.J. & RATE, A.W. 1987. Aluminium-tannin equilibria: a potentiometric study. *Australian Journal of Chemistry*, **40**, 2015-2022.

POWELL, H.K.J. & FLORENCE, T.M. 1990. Effect of mercury(II) on metal complex labilities determined by direct-current anodic stripping voltammetry on a thin mercury film electrode. *Analytica Chimica Acta*, **228**, 327-331.

PRAKASH, A. & MacGREGOR, D.J. 1983. Environmental and human health significance of humic materials: an overview. In: *Aquatic and Terrestrial Humic Materials*. R.F. Christman & E.T. Gjessing (eds), Ann Arbor Science, Ann Arbor, pp 481-494.

PRESTON, C.M. & SCHNITZER, M. 1984. Effects of chemical modifications and extractants on the carbon-13 NMR spectra of humic materials. *Soil Science Society of America Journal*, **48**, 305-311.

PRESTON, C.M. & BLACKWELL, B.A. 1985. Carbon-13 nuclear magnetic resonance for a humic acid and a fulvic acid: signal-to-noise optimization, quantitation, and spin-echo techniques. *Soil Science*, **139**, 88-96.

PURDIE, N., TOMSON, M.B. & RIEMANN, N. 1972. The thermodynamics of ionization of polycarboxylic acids. *Journal of Solution Chemistry*, **1**, 465-476.

RAINVILLE, D.P. & WEBER, J.H. 1982. Complexing capacity of soil fulvic acid for Cu^{2+}, Cd^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} measured by dialysis titration: a model based on soil fulvic acid aggregation. *Canadian Journal of Chemistry*, **60**, 1-5.

RAJAN, K.S. & MARTELL, A.E. 1967. Polymeric copper(II) complexes of hydroxy acids. *Journal of Inorganic and Nuclear Chemistry*, **29**, 463-471.

RAMAMOORTHY, S., MANNING, P.G. & GUARNASCHELLI, C. 1972. Equilibrium studies of metal-ion complexes of interest to natural waters - IV. Simple and mixed Cu(II)-carboxylic complexes involving nitrilotriacetic acid and secondary ligand. *Journal of Inorganic and Nuclear Chemistry*, **34**, 3443-3448.

RAMUNNI, A.U. & PALMIERI, F. 1985. Use of ultrasonic treatment for extraction of humic acid with inorganic reagents from soil. *Organic Geochemistry*, **8**, 241-246.

RASPOR, B. & VALENTA, P. 1988. Adsorption of humic substances isolated from marine and estuarine sediments. *Marine Chemistry*, **25**, 211-226.

RAVICHANDRAN, K., LEWIS, J.J., YIN, I-H., KOENIGBAUER, M., POWLEY, C.R., SHAH, P. & ROGERS, L.B. 1988. Computer-controlled linear pH gradient for high-performance liquid chromatographic fractionations of aromatic carboxylic acids and of humic and fulvic acids. *Journal of Chromatography*, **439**, 213-226.

RECHNITZ, G.A. & ZAMOCHNICK, S.B. 1964. Application of cation-sensitive glass electrodes to the study of alkali metal complexes - II. Use of a potential comparison method. *Talanta*, **11**, 1061-1065.

RECKHOW, D.A., SINGER, P.C. & MALCOLM, R.L. 1990. Chlorination of humic materials: byproduct formation and chemical interpretations. *Environmental Science and Technology*, **24**, 1655-1664.

REID, P.M., WILKINSON, A.E., TIPPING, E. & JONES, M.N. 1990. Determination of molecular weights of humic substances by analytical (UV scanning) ultracentrifugation. *Geochimica et Cosmochimica Acta*, **54**, 131-138.

REUTER, J.H., GHOSAL, M., CHIAN, E.S.K. & GIABBAI, M. 1983. Oxidative degradation studies on aquatic humic substances. In: *Aquatic and Terrestrial Humic Materials*. R.F. Christman & E.T. Gjessing (eds), Ann Arbor Science, Ann Arbor, pp 107-125.

RICE, J.A. & MacCARTHY, P. 1989a. Characterisation of a stream sediment humin. In: *Aquatic Humic Substances. Influence on Fate and Treatment of Pollutants*. I.H. Suffet & P. MacCarthy (eds), American Chemical Society, Advances in Chemistry Series 219, Washington DC, pp 41-54.

RICE, J.A. & MacCARTHY, P. 1989b. Isolation of humin by liquid-liquid partitioning. *The Science of the Total Environment*, **81/82**, 61-69.

RICE, R.J., PONTIKOS, N.M. & McCREERY, R.L. 1990. Quantitative correlations of heterogeneous electron-transfer kinetics with surface properties of glassy carbon electrodes. *Journal of the American Chemical Society*, **112**, 4617-4622.

RIFFALDI, R. & SCHNITZER, M. 1973. Effects of 6 N HCl hydrolysis on the analytical characteristics and chemical structure of humic acids. *Soil Science*, **115**, 349-356.

RITCHIE, G.S.P. & POSNER, A.M. 1982. The effect of pH and metal binding on the transport properties of humic acids. *Journal of Soil Science*, **33**, 233-247.

ROBINSON, R.A. & STOKES, R.H. 1959. *Electrolyte Solutions*. Butterworth, London.

ROEMELT, P.M. & SEITZ, W.R. 1982. Fluorescence polarization studies of perylene-fulvic acid binding. *Environmental Science and Technology*, **16**, 613-616.

ROSSELAND, B.O., ELDHUSET, T.D. & STAURNES, M. 1990. Environmental effects of aluminium. *Environmental Geochemistry and Health*, **12**, 17-27.

RUGGIERO, P., INTERESSE, F.S. & SCIACOVELLI, O. 1979. [^{1}H] and [^{13}C] NMR studies on the importance of aromatic structures in fulvic and humic acids. *Geochimica et Cosmochimica Acta*, **43**, 1771-1775.

RUSSELL, J.M. 1977. *A Thermodynamic Study of Transition Metal Oxime Complexes*. Ph. D. Thesis, University of Canterbury, New Zealand.

RUZIC, I. 1984. Kinetics of complexation and determination of complexation parameters in natural waters. In: *Complexation of Trace Metals in Natural Waters*. C.J.M. Kramer & J.C. Duinker (eds), Martinus Nijhoff/Dr W. Junk, The Hague, pp 131-147.

RYAN, D.K., VENTRY, L.S., CABANISS, S.E. & SHUMAN, M.S. 1990. Exchange of comments on fluorescence quenching measurements on copper-fulvic acid binding. *Analytical Chemistry*, **62**, 1523-1526.

SAAR, R.A. & WEBER, J.H. 1982. Fulvic acid: modifier of metal-ion chemistry. *Environmental Science and Technology*, **16**, 510A-517A.

SADA, A., DI PASCALE, G. & CACACE, M.G. 1979. Salt effects on adsorption of aromatic compounds in Sephadex G-25 chromatography. *Journal of Chromatography*, **177**, 353-356.

SAGBERG, P. & LUND, W. 1982. Trace metal analysis by anodic-stripping voltammetry. Effect of surface-active substances. *Talanta*, **29**, 457-460.

SAIZ-JIMENEZ, C. & DE LEEUW, J.W. 1987a. Nature of plant components identified in soil humic acids. *The Science of the Total Environment*, **62**, 115-119.

SAIZ-JIMENEZ, C. & DE LEEUW, J.W. 1987b. Chemical structure of a soil humic acid as revealed by analytical pyrolysis. *Journal of Analytical and Applied Pyrolysis*, **11**, 367-376.

SAKAI, Y. 1980. Photometric determination of copper with N-(dithiocarboxy)sarcosine after preconcentration with Amberlite XAD-2 resin. *Talanta*, **27**, 1073-1076.

SANDERS, J.R. & BLOOMFIELD, C. 1980. The influence of pH, ionic strength and reactant concentrations on copper complexing by humified organic matter. *Journal of Soil Science*, **31**, 53-63.

SATO, T., OSE, Y., NAGASE, H. & HAYASE, K. 1987. Adsorption of mutagens by humic acid. *The Science of the Total Environment*, **62**, 305-310.

SCHNITZER, M., WRIGHT, J.R. & DESJARDIN, J.G. 1958. A comparison of the effectiveness of various extractants for organic matter from two horizons of a Podzol profile. *Canadian Journal of Soil Science*, **38**, 49-53.

SCHNITZER, M. & OGNER, G. 1970. The occurrence of fatty acids in fulvic acid, a soil humic fraction. *Israel Journal of Chemistry*, **8**, 505-512.

SCHNITZER, M. & HANSEN, E.H. 1970. Organo-metallic interactions in soils. 8. An evaluation of methods for the determination of stability constants of metal-fulvic acid complexes. *Soil Science*, **109**, 333-340.

SCHNITZER, M. & NEYROUD, J.A. 1975. Alkanes and fatty acids in humic substances. *Fuel*, **54**, 17-19.

SCHNITZER, M. & DE SERRA, M.I.O. 1975. The chemical degradation of a humic acid. *Canadian Journal of Chemistry*, **51**, 1554-1566.

SCHNITZER, M. 1977. Recent findings on the characterization of humic substances extracted from soils from widely differing climatic zones. In: *Soil Organic Matter Studies, Vol II*, IAEA, Austria, pp 117-132.

SCHNITZER, M. & KERNDORFF, H. 1981. Reactions of fulvic acid with metal ions. *Water, Air, and Soil Pollution*, **15**, 97-108.

SCHNITZER, M. & PRESTON, C.M. 1983. Effects of acid hydrolysis on the ^{13}C NMR spectra of humic substances. *Plant and Soil*, **75**, 201-211.

SCHNITZER, M. 1985. Nature of nitrogen in humic substances. In: *Humic Substances in Soil, Sediment, and Water. Geochemistry, Isolation, and Characterization*. G.R. Aiken, D.M. McKnight, R.L. Wershaw & P. MacCarthy (eds), Wiley-Interscience, New York, pp 303-325.

SCHNITZER, M. & FARMER, V.C. 1985. Aromaticity of soil fulvic acid. *Nature*, **316**, 658.

SCHNITZER, M. & PRESTON, C.M. 1986. Analysis of humic acids by solution and solid-state carbon-13 nuclear magnetic resonance. *Soil Science Society of America Journal*, **50**, 326-331.

SCHNITZER, M. & SCHUPPLI, P. 1989a. The extraction of organic matter from selected soils and particle size fractions with 0.5 M NaOH and 0.1 M $Na_4P_2O_7$ solutions. *Canadian Journal of Soil Science*, **69**, 253-262.

SCHNITZER, M. & SCHUPPLI, P. 1989b. Method for the sequential extraction of organic matter from soils and soil fractions. *Soil Science Society of America Journal*, **53**, 1418-1424.

SCHÖNBERGER, E.A. & PICKERING, W.F. 1980. The influence of pH and complex formation on the ASV peaks of Pb, Cu and Cd. *Talanta*, **27**, 11-18.

SCHULTEN, H-R., ABBT-BRAUN, G. & FRIMMEL, F.H. 1987. Time-resolved pyrolysis field ionization mass spectrometry of humic material isolated from freshwater. *Environmental Science and Technology*, **21**, 349-357.

SEDLACEK, J., GJESSING, E.T. & KALLQVIST, T. 1989. Influence of different aquatic humus fractions on uptake of cadmium to alga *Selenastrum capricornutum* Printz. *The Science of the Total Environment*, **81/82**, 711-718.

SEKERKA, I. & LECHNER, J.F. 1978. Response of copper(II) selective electrode to some complexing agents. *Analytical Letters*, **A11**, 415-427.

SENESI, N., CHEN, Y. & SCHNITZER, M. 1977. Aggregation-dispersion phenomena in humic substances. In: *Soil Organic Matter Studies Vol II*. IAEA, Austria, pp 143-155.

SENESI, N., TESTINI, C. & POLEMIO, M. 1983. Chemical and spectroscopic characterization of soil organic matter fractions isolated by sequential extraction procedure. *Journal of Soil Science*, **34**, 801-813.

SENESI, N., BOCIAN, D.F. & SPOSITO, G. 1985. Electron spin resonance investigation of copper(II) complexation by fulvic acid extracted from sewage sludge. *Soil Science of America Journal*, **49**, 119-126.

SENESI, N. 1986. Comparative electron spin resonance study of copper(II) complexes with fulvic acids and model organic compounds. In: *Proceedings of the International Conference on Chemicals in the Environment*. J.N. Lester, R. Perry & R.M. Sterritt (eds), Selper Ltd, London, pp 614-621.

SENESI, N., SPOSITO, G., HOLTZCLAW, K.M. & BRADFORD, G.R. 1989. Chemical properties of metal-humic acid fractions of a sewage sludge-amended Aridisol. *Journal of Environmental Quality*, **18**, 186-194.

SENESI, N. 1990. Molecular and quantitative aspects of the chemistry of fulvic acid and its interactions with metal ions and organic chemicals. Part II. The fluorescence spectroscopy approach. *Analytica Chimica Acta*, **232**, 77-106.

SEQUI, P., GUIDI, G. & PETRUZELLI, G. 1975. Influence of metals on solubility of soil organic matter. *Geoderma*, **13**, 153-161.

SERKIZ, S.M. & PERDUE, E.M. 1990. Isolation of dissolved organic matter from the Suwannee River using reverse osmosis. *Water Research*, **24**, 911-916.

SERVOS, M.R. & MUIR, D.C.G. 1989. Effect of dissolved organic matter from Canadian Shield lakes on the bioavailability of 1,3,6,8-tetrachlorodibenzo-p-dioxin to the amphipod *Crangonyx laurentianus*. *Environmental Toxicology and Chemistry*, **8**, 141-150.

SHANMUKHAPPA, H., BANERJEE, D.K. & KRISHNAMURTHY, K. 1986. Humic acids, copper and iron in the sediments of Porto Novo, India. In: *Proceeding of the International Conference on Chemicals in the Environment*. J.N. Lester, R. Perry & R.M. Sterritt (eds), Selper, London, pp 800-808.

SHINOZUKA, N., LEE, C. & HAYANO, S. 1987. Solubilizing action of humic acid from marine sediment. *The Science of the Total Environment*, **62**, 311-314.

SHUMAN, M.S., WEBER, J.H. & CABANISS, S.E. Experimental methods for studying metal-humic coordination. In: *Humic Substances in Soil Sediment, and Water: Structures and Interactions*. M.H.B. Hayes, P. MacCarthy, R.L. Malcolm & R.S. Swift (eds), Wiley, New York (in press).

SIKORA, F.J. & STEVENSON, F.J. 1988. Silver complexation by humic substances: conditional stability constants and nature of reactive sites. *Geoderma*, **42**, 353-363.

SIMONART, P., BATISTIC, L. & MAYAUDON, J. 1967. Isolation of protein from humic acid extracted from soil. *Plant and Soil*, **27**, 153-161.

SJÖBERG, S. & ÖHMAN, L-O. 1985. Equilibrium and structural studies of silicon(IV) and aluminium(III) in aqueous solution. Part 13. A potentiometric and ^{27}Al nuclear magnetic resonance study of speciation and equilibria in the aluminium(III)-oxalic acid-hydroxide system. *Journal of the Chemical Society - Dalton Transactions*, 2665-2669.

SKOGERBOE, R.K., WILSON, S.A., OSTERYOUNG, J.G., FLORENCE, T.M. & BATLEY, G.E. 1980. Exchange of comments on scheme for classification of heavy metal species in natural waters. *Analytical Chemistry*, **52**, 1960-1963.

SKOGERBOE, R.K. & WILSON, S.A. 1981. Reduction of ionic species by fulvic acid. *Analytical Chemistry*, **53**, 228-232.

SLAVEK, J., WOLD, J. & PICKERING, W.F. 1982. Selective extraction of metal ions associated with humic acids. *Talanta*, **29**, 743-749.

SLAWINSKA, D. & SLAWINSKI, J. 1975a. Spectroscopic study on mild oxidation of humus acids. I. Oxidation with molecular oxygen. *Polish Journal of Soil Science*, **8**, 37-47.

SLAWINSKA, D. & SLAWINSKI, J. 1975b. Spectroscopic study on mild oxidation of humus acids. II. Influence of light on oxidation with molecular oxygen. *Polish Journal of Soil Science*, **8**, 49-58.

SMART, R.B. & STEWART, E.E. 1985. Differential pulse anodic stripping voltammetry of cadmium(II) at a membrane-covered electrode: measurement in the presence of model organic compounds. *Environmental Science and Technology*, **19**, 137-140.

SMITH, J.A., WITOWSKI, P.J. & CHIOU, C.T. 1988. Partition of nonionic organic compounds in aquatic systems. *Reviews of Environmental Contamination and Technology*, **103**, 127-151.

SMITH, R.M., MARTELL, A.E. & MOTEKAITIS, R.J. 1985. Prediction of stability constants. I. Protonation constants of carboxylates and formation constants of their complexes with Class A metal ions. *Inorganica Chimica Acta*, **99**, 207-216.

SÖCHTIG, H. 1972. Gel chromatography as a method for characterization of humic systems. In: *Humic Substances, Their Structure and Function in the Biosphere*. D. Povoledo & H.L. Golterman (eds), Wageningen, pp 321-335

SOHN, M. & WEESE, D. 1986. ^{13}C NMR spectra and Cu(II) formation constants for humic acids from fluvial, estuarine and marine sediments. *Marine Chemistry*, **20**, 61-72.

SOJO, L.E., GAMBLE, D.S., LANGFORD, C.H. & ZIENIUS, R.H. 1989. The reactions of paraquat and divalent metal ions with humic acid: factors influencing stoichiometry. *Journal of Environmental Science and Health*, **B24**, 619-646.

SPOSITO, G. & HOLTZCLAW, K.M. 1977. Titration studies on the polynuclear, polyacidic nature of fulvic acid extracted from sewage sludge-soil mixtures. *Soil Science Society of America Journal*, **41**, 330-336.

SPOSITO, G., HOLTZCLAW, K.M. & KEECH, D.A. 1977. Proton binding in fulvic acid extracted from sewage sludge-soil mixtures. *Soil Science Society of America Journal*, **41**, 1119-1125.

SPOSITO, G. 1981. Trace metals in contaminated waters. *Environmental Science and Technology*, **15**, 396-403.

SPOSITO, G. 1986. Sorption of trace metals by humic materials in soils and natural waters. *CRC Critical Reviews in Environmental Control*, **16**, 193-229.

STACKHOUSE, R.A. & BENSON, W.H. 1988. The influence of humic acid on the toxicity and bioavailability of selected trace metals. *Aquatic Toxicology*, **13**, 99-108.

STACKHOUSE, R.A. & BENSON, W.H. 1989. Interaction of humic acid with selected trace metals: influence on bioaccumulation in Daphnids. *Environmental Toxicology and Chemistry*, **8**, 639-644.

STAUBER, J.L. & FLORENCE, T.M. 1985a. Interactions of copper and manganese: A mechanism by which manganese alleviates copper toxicity to the marine diatom, *Nitzschia closterium* (Ehrenberg) W. Smith. *Aquatic Toxicology*, **7**, 241-254.

STAUBER, J.L. & FLORENCE, T.M. 1985b. The influence of iron on copper toxicity to the marine diatom *Nitzschia closterium* (Ehrenberg) W. Smith. *Aquatic Toxicology*, **6**, 297-305.

STAUBER, J.L. & FLORENCE, T.M. 1986. Reversibility of copper-thiol binding in *Nitzschia closterium* and *Chlorella pyrenoidosa*. *Aquatic Toxicology*, **8**, 223-229.

STAUBER, J.L. & FLORENCE, T.M. 1987. Mechanism of toxicity of ionic copper and copper complexes to algae. *Marine Biology*, **94**, 511-519.

STAUBER, J.L. & FLORENCE, T.M. 1989. The effect of culture medium on metal toxicity to the marine diatom *Nitzschia closterium* and the freshwater green alga *Chlorella pyrenoidosa*. *Water Research*, **23**, 907-911.

STAUBER, J.L. & FLORENCE, T.M. 1990. Fumed silica for the direct determination of lead in urine by differential-pulse anodic stripping voltammetry. *Analytica Chimica Acta*, **237**, 177-180.

STEELINK, C. & PETSOM, A. 1987. Structural features of lignins and humic substances as revealed by spin-echo and broadband decoupled C-13 NMR spectroscopy. *The Science of the Total Environment*, **62**, 165-174.

STEVENSON, F.J. 1977. Nature of divalent transition metal complexes of humic acids as revealed by a modified potentiometric titration method. *Soil Science*, **123**, 10-17.

STEVENSON, F.J. 1982. *Humus Chemistry*, Wiley, New York.

STEVENSON, F.J. & VANCE, G.F. 1989. Naturally occurring aluminum-organic complexes. In: *The Environmental Chemistry of Aluminum*. G. Sposito (ed), CRC Press, Florida, pp 117-145.

STOJEK, Z., STEPNIK, B. & KUBLIK, Z. 1976. Cyclic and stripping voltammetry with graphite based thin mercury film electrodes prepared "in situ". *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **74**, 277-295.

STOLZBERG, R.J. 1977. Potential inaccuracy in trace metal speciation measurements by differential pulse polarography. *Analytica Chimica Acta*, **92**, 193-196.

STUERMER, D.H. & PAYNE, J.R. 1976. Investigation of seawater and terrestrial humic substances with carbon-13 and proton nuclear magnetic resonance. *Geochimica et Cosmochimica Acta*, **40**, 1109-1114.

STULIKOVA, M. 1973. The deposition and stripping of mercury on a glassy carbon rotating disk electrode. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **48**, 33-45.

SUGIMURA, Y. & SUZUKI, Y. 1988. A high-temperature catalytic oxidation method for the determination of non-volatile dissolved organic carbon in seawater by direct injection of a liquid sample. *Marine Chemistry*, **24**, 105-131.

SUSETYO, W., DOBBS, J.C., CARREIRA, L.A., AZARRAGA, L.V. & GRIMM, D.M. 1990. Development of a statistical model for metal-humic interactions. *Analytical Chemistry*, **62**, 1215-1221.

SWIFT, R.S., THORNTON, B.K. & POSNER, A.M. 1970. Spectral characteristics of a humic acid fractionated with respect to molecular weight using an agar gel. *Soil Science*, **110**, 93-99.

SWIFT, R.S. & POSNER, A.M. 1971. Gel chromatography of humic acid. *Journal of Soil Science*, **22**, 237-249.

SWIFT, R.S. & POSNER, A.M. 1972. Autoxidation of humic acid under alkaline conditions. *Journal of Soil Science*, **23**, 381-393.

SYLVA, R.N. & DAVIDSON, M.R. 1979. The hydrolysis of metal ions. Part 1. Copper(II). *Journal of the Chemical Society - Dalton Transactions*, 232-235.

SZENTIRMAY, M.N. & MARTIN, C.R. 1984. Ion-exchange selectivity of Nafion films on electrode surfaces. *Analytical Chemistry*, **56**, 1898-1902.

TABOR, M.W. & LOPER, J.C. 1985. Analytical isolation, separation and identification of mutagens from nonvolatile organics of drinking water. *International Journal of Environmental Analytical Chemistry*, **19**, 281-318.

TALIAFERRO, C.H., MOTEKAITIS, R.J. & MARTELL, A.E. 1984. New multidentate ligands. 22. N,N'-dipyridoxylethylenediamine-N,N'-diacetic acid: a new chelating ligand for trivalent metal ions. *Inorganic Chemistry*, **23**, 1188-1192.

TAM, S-C. & McCOLL, J.G. 1990. Aluminum- and calcium-binding affinities of some organic ligands in acidic conditions. *Journal of Environmental Quality*, **19**, 514-520.

TANFORD, C. 1973. *The Hydrophobic Effect*, Wiley, New York.

TAY, E.B-T., KHOO, S-B. & ANG, S-G. 1989. Oxygen removal in flow injection anodic stripping voltammetry. *Analyst*, **114**, 1271-1273.

TAYLOR, M.C. 1980. *Podzols: Aspects of Their Chemistry and Development*. Ph. D. thesis, University of Canterbury, New Zealand.

TEASDALE, R.D. 1987. Copper-induced indefinite aggregation of humic substances: theoretical consequences for copper-binding behaviour. *Journal of Soil Science*, **38**, 433-442.

TEGELAAR, E.W., DE LEEUW, J.W. & SAIZ-JIMENEZ, C. 1989. Possible origin of aliphatic moieties in humic substances. *The Science of the Total Environment*, **81/82**, 1-18.

TERCIER, M.-L., BERNARD, C., BUJARD, F., RODAK, S. & BUFFLE, J. 1990. Photomicroscopic measurement of the diffusion layer around mercury drop electrodes: Part I, Description of the electrochemical system. *Electroanalysis*, **2**, 89-97.

TERCIER, M.-L. & BUFFLE, J. 1990. Photomicroscopic measurement of the diffusion layer around mercury drop electrodes: Part II, Choice of the optimal conditions. *Electroanalysis*, **2**, 99-105.

THOMANN, R.V. 1989. Bioaccumulation model of organic chemical distribution in aquatic food chains. *Environmental Science and Technology*, **23**, 669-707.

THORN, K.A. 1987. Structural characteristics of the IHSS Suwannee River fulvic and humic acids determined by solution state C-13 NMR spectroscopy. *The Science of the Total Environment*, **62**, 175-183.

THORN, K.A., RICE, J.A., WERSHAW, R.L. & MacCARTHY, P. 1987a. C-13 NMR characterization of humic materials isolated by an MIBK partitioning procedure. *The Science of the Total Environment*, **62**, 185-188.

THORN, K.A., STEELINK, C. & WERSHAW, R.L. 1987b. Methylation patterns of aquatic humic substances determined by ^{13}C NMR spectroscopy. *Organic Geochemistry*, **11**, 123-137.

THURMAN, E.M., MALCOLM, R.L. & AIKEN, G.R. 1978. Prediction of capacity factors for aqueous organic solutes adsorbed on a porous acrylic resin. *Analytical Chemistry*, **50**, 775-779.

THURMAN, E.M. & MALCOLM, R.L. 1979. Concentration and fractionation of hydrophobic organic acid constituents from natural waters by liquid chromatography. *United States Geological Survey Water-Supply Paper*, **1817-G**, 16 pp.

THURMAN, E.M. & MALCOLM, R.L. 1981. Preparative isolation of aquatic humic substances. *Environmental Science and Technology*, **15**, 463-466.

THURMAN, E.M., WERSHAW, R.L., MALCOLM, R.L. & PINCKNEY, D.J. 1982. Molecular size of aquatic humic substances. *Organic Geochemistry*, **4**, 27-35.

THURMAN, E.M. 1985. *Organic Geochemistry of Natural Waters*. Martinus Nijhoff/ Dr W. Junk Publishers, Dordrecht.

THURMAN, E.M. & FIELD, J. 1989. Separation of humic substances and anionic surfactants from ground water by selective adsorption. In: *Aquatic Humic Substances. Influence on Fate and Treatment of Pollutants*. I.H. Suffet & P. MacCarthy (eds), Advances in Chemistry Series, Vol 219, American Chemical Society, Washington DC, pp 107-114.

THURMAN, E.M. & MALCOLM, R.L. 1989. Nitrogen and amino acids in fulvic and humic acids from the Suwannee River. In: *Humic Substances in the Suwannee River, Georgia: Interactions, Properties, and Proposed Structures*. R.C. Averett, J.A. Leenheer, D.M. McKnight & K.A. Thorn (eds), U.S. Geological Survey Open-File Report, **87-557**, pp 99-118.

TINSLEY, J. & SALAM, A. 1961. Extraction of soil organic matter with aqueous solvents. *Soils and Fertilizers*, **14**, 81-84.

TIPPING, E. 1981. The adsorption of aquatic humic substances by iron oxides. *Geochimica et Cosmochimica Acta*, **45**, 191-199.

TIPPING, E. & OHNSTAD, M. 1984. Aggregation of aquatic humic substances. *Chemical Geology*, **44**, 349-357.

TIPPING, E. & BACKES, C.A. 1988. Organic complexation of Al in acid waters: model-testing by titration of a streamwater sample. *Water Research*, **22**, 593-595.

TIPPING, E., WOOF, C., BACKES, C.A. & OHNSTAD, M. 1998a. Aluminium speciation in acidic natural waters: testing of a model for Al-humic complexation. *Water Research*, **22**, 321-326.

TIPPING, E., BACKES, C.A. & HURLEY, M.A. 1988b. The complexation of protons, aluminium and calcium by aquatic humic substances: a model incorporating binding site heterogeneity and macroionic effects. *Water Research*, **22**, 597-611.

TIPPING, E., REDDY, M.M. & HURLEY, M.A. 1990. Modeling electrostatic and heterogeneity effects on proton dissociation from humic substances. *Environmental Science and Technology*, **24**, 1700-1705.

TOWN, R.M. & POWELL, H.K.J. 1989. Interaction of humic acid with hydrophobic metal complexes. In: *Trace Elements in New Zealand: Environmental, Human and Animal*. Proceedings of the New Zealand Trace Elements Group Conference, Lincoln College, Canterbury, New Zealand (30 Nov - 2 Dec 1988), pp 79-84.

TRAINA, S.J., SPONTAK, D.A. & LOGAN, T.J. 1989. Effects of cations on complexation of naphthalene by water-soluble carbon. *Journal of Environmental Quality*, **18**, 221-227.

TRUITT, R.E. & WEBER, J.H. 1981. Determination of complexing capacity of fulvic acid for copper(II) and cadmium(II) by dialysis titration. *Analytical Chemistry*, **53**, 337-342.

TSCHAPEK, M., WASOWSKI, C. & TORRES SANCHEZ, R.M. 1981. Humic acid as a colloidal surfactant. *Plant and Soil*, **63**, 261-271.

TURNER, D.R. & WHITFIELD, M. 1980. Chemical definition of the biologically available fraction of trace metals in natural waters. *Thalassia Jugoslavica*, **16**, 231-241.

TURNER, D.R. 1984. Relationships between biological availability and chemical measurements. *Metal Ions in Biological Systems*, **18**, 137-164.

TURNER, D.R., VARNEY, M.S., WHITFIELD, M., MANTOURA, R.F.C. & RILEY, J.P. 1986. Electrochemical studies of copper and lead complexation by fulvic acid. I. Potentiometric measurements and a critical comparison of metal binding models. *Geochimica et Cosmochimica Acta*, **50**, 289-297.

TUSCHALL, J.R. Jr. & BREZONIK, P.L. 1980. Characterization of organic nitrogen in natural waters: its molecular size, protein content, and interactions with heavy metals. *Limnology and Oceanography*, **25**, 495-504.

TUSCHALL, J.R. Jr. & BREZONIK, P.L. 1983a. Complexation of heavy metals by aquatic humus: a comparative study of five analytical methods. In: *Aquatic and Terrestrial Humic Materials*. R.F. Christman & E.T. Gjessing (eds), Ann Arbor Science, Ann Arbor, pp 275-294.

TUSCHALL, J.R. Jr. & BREZONIK, P.L. 1983b. Application of continuous-flow ultrafiltration and competing ligand/differential spectophotometry for measurement of heavy metal complexation by dissolved organic matter. *Analytica Chimica Acta*, **149**, 47-58.

TUSCHALL, J.R. Jr. & BREZONIK, P.L. 1984. Analytical methods for measurement and interpretation of metal binding by aquatic humus and model compounds. In: *Complexation of Trace Metals in Natural Waters*. C.J.M. Kramer & J.C.Duinker (eds), Martinus Nijhoff/Dr W. Junk, The Hague, pp 83-94.

UGAPO, T. & PICKERING, W.F. 1985. Effect of organic colloids on ASV signals of Cd, Pb and Cu. *Talanta*, **32**, 131-138.

UGO, P., BALLARIN, B., DANIELE, S. & MAZZOCCHIN, G.A. 1990. Electrochemistry of Yb^{3+} and Eu^{3+} at Nafion modified electrodes. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **291**, 187-199.

ULRICH, H-J., STUMM, W. & COSOVIC, B. 1988. Adsorption of aliphatic fatty acids on aquatic interfaces. Comparison between two model surfaces: the mercury electrode and δ-Al_2O_3 colloids. *Environmental Science and Technology*, **22**, 37-41.

UPADHYAY, P.K. 1989. A simple procedure for activating a glassy carbon electrode. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **271**, 339-343.

VANCE, G.F., BOYD, S.A. & MOKMA, D.L. 1985. Extraction of phenolic compounds from a Spodosol profile: an evaluation of three extractants. *Soil Science*, **140**, 412-420.

VAN DEN BERG, C.M.G., WONG, P.T.S. & CHAU, Y.K. 1979. Measurement of complexing materials excreted from algae and their ability to ameliorate copper toxicity. *Journal. Fisheries Research Board of Canada*, **36**, 901-905.

VAN DEN HOOP, M.A.G.T., VAN LEEUWEN, H.P. & CLEVEN, R.F.M.J. 1990. Study of the polyelectrolyte properties of humic acids by conductimetric titration. *Analytica Chimica Acta*, **232**, 141-148.

VAN LEEUWEN, H.P. 1984. Influence of reactant adsorption on limiting currents in normal pulse polarography. Part III. Experimental illustration of some basic effects for the reduction of methylene blue and lead(II) in humic acid at the SMDE. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **162**, 67-76.

VAN LEEUWEN, H.P. 1987. Voltammetric titrations involving metal complexes: effect of kinetics and diffusion coefficients. *The Science of the Total Environment*, **60**, 45-55.

VAN LEEUWEN, H.P. CLEVEN, R.F.M.J. & BUFFLE, J. 1989a. Voltammetric techniques for complexation measurements in natural aquatic media. Role of the size of macromolecular ligands and dissociation kinetics of complexes. *Pure and Applied Chemistry*, **61**, 255-274.

VAN LEEUWEN, H.P., DE JONG, H.G. & HOLUB, K. 1989b. Voltammetry of metal complex systems with different diffusion coefficient of the species involved. Part IV. Simulation of the limiting current for any metal-to-ligand ratio and elaboration to voltammetric titration curves. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **260**, 213-220.

VAN NOORT, P., LAMMERS, R., VERBOOM, H. & WONDERGEM, E. 1988. Rates of triplet humic acid sensitized photolysis of hydrophobic compounds. *Chemosphere*, **17**, 35-38.

VARNEY, M.S., MANTOURA, R.F.C., WHITFIELD, M., TURNER, D.R. & RILEY, J.P. 1983. Potentiometric and conformational studies of the acid-base properties of fulvic acid from natural waters. In: *Trace Metals in Seawater*. C.S. Wong, E. Boyle, K.W. Bruland, J.D. Burton & E.D. Goldberg (eds), NATO Conference Series, Series IV: Marine Sciences, Plenum, New York, pp 751-772.

VARNEY, M.S., TURNER, D.R., WHITFIELD, M. & MANTOURA, R.F.C. 1984. The use of electrochemical techniques to monitor complexation capacity titrations in natural waters. In: *Complexation of Trace Metals in Natural Waters*. C.J.M. Kramer & J.C.Duinker (eds), Martinus Nijhoff/Dr W. Junk, The Hague, pp 33-46.

VARTIAINEN, T., LIIMATAINEN, A., JÄÄSKELÄINEN, S. & KAURANEN, P. 1987. Comparison of solvent extractions and resin adsorption for isolation of mutagenic compounds from chlorinated drinking water with high humus content. *Water Research*, **21**, 773-779.

VASCONCELOS, M.T.S.D., SANTOS, A.P.L.M.G. & MACHADO, A.A.S.C. 1989. Evidence of conformational changes in fulvic acids from dialysis. *The Science of the Total Environment*, **81/82**, 489-500.

VASSALLO, A.M., WILSON, M.A., COLLIN, P.J., OADES, J.M., WATERS, A.G. & MALCOLM, R.L. 1987. Structural analysis of geochemical samples by solid-state nuclear magnetic resonance spectrometry. Role of paramagnetic material. *Analytical Chemistry*, **59**, 558-562.

VASSOS, B.H. & MARK, H.B. Jr. 1967. The anodic dissolution of thin films of copper metal from pyrolytic graphite. A study of the multiple dissolution current peaks. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **13**, 1-9.

VISSER, S.A. 1983. Comparative study on the elementary composition of fulvic and humic acids of aquatic origin and from soils and microbial substrates. *Water Research*, **17**, 1393-1396.

VISSER, S.A. 1988. Effects of humic substances on higher animals and man: the possible use of humic compounds in medical treatments. In: *Abstracts of Oral and Poster Papers*. IHSS Fourth International Meeting, Matalascañas, Spain.

VOGEL, A.I. 1974. *Textbook of Quantitative Inorganic Analysis*. Longman, London.

WAGEMANN, R. 1980. Cupric ion-selective electrode and inorganic cationic complexes of copper. *Journal of Physical Chemistry*, **84**, 3433-3436.

WAITE, T.D. & MOREL, F.M.M. 1983. Characterization of complexing agents in naturals waters by copper(II)/copper(I) amperometry. *Analytical Chemistry*, **55**, 1268-1274.

WAITE, T.D. & MOREL, F.M.M. 1984. Ligand exchange and fluorescence quenching studies of the fulvic acid-iron interaction. Effects of pH and light. *Analytica Chimica Acta*, **162**, 263-274.

WAITE, T.D. 1988. Photochemical effects on the mobility and fate of heavy metals in the aquatic environment. *Environmental Technology Letters*, **9**, 977-982.

WALLER, F.J. 1989. Fluoropolymers. *Journal of Chemical Education*, **66**, 487-488.

WAN, C-C., CHIANG, S. & CORSINI, A. 1985. Two-column method for preconcentration of trace metals in natural waters on acrylate resin. *Analytical Chemistry*, **57**, 719-723.

WANG, J. & LUO, D-B. 1984. Effect of surface-active compounds on voltammetric stripping analysis at the mercury film electrode. *Talanta*, **31**, 703-707.

WANG, J. & HUTCHINS-KUMAR, L.D. 1986. Cellulose acetate coated mercury film electrodes for anodic stripping voltammetry. *Analytical Chemistry*, **58**, 402-407.

WANG, J. & TUZHI, P. 1986. Enhanced stability of glassy carbon detectors following a simple electrochemical pretreatment. *Analytical Chemistry*, **58**, 1787-1790.

WANG, J. & LIN, M.S. 1988. In situ electrochemical renewal of glassy carbon electrodes. *Analytical Chemistry*, **60**, 499-502.

WANG, Z-D., PANT, B.C. & LANGFORD, C.H. 1990. Spectroscopic and structural characterization of a Laurentian fulvic acid: notes on the origin of the color. *Analytica Chimica Acta*, **232**, 43-49.

WERSHAW, R.L., BURCAR, P.J. & GOLDBERG, M.C. 1969. Interaction of pesticides with natural organic matter. *Environmental Science and Technology*, **3**, 271-273.

WERSHAW, R.L. & PINCKNEY, D.J. 1973a. The fractionation of humic acids from natural water systems. *Journal of Research. U.S. Geological Survey*, **1**, 361-366.

WERSHAW, R.L. & PINCKNEY, D.J. 1973b. Determination of the association and dissociation of humic acid fractions by small angle x-ray scattering. *Journal of Research. U.S. Geological Survey*, **1**, 701-707.

WERSHAW, R.L., PINCKNEY, D.J. & BOOKER, S.E. 1977. Chemical structure of humic acids - Part 1. A generalized structural model. *Journal of Research. U.S. Geological Survey*, **5**, 565-569.

WERSHAW, R.L. & PINCKNEY, D.J. 1977. Chemical structure of humic acids - Part 2. The molecular aggregation of some humic acid fractions in N,N-dimethylformamide. *Journal of Research. U.S. Geological Survey*, **5**, 571-577.

WERSHAW, R.L. & PINCKNEY, D.J. 1980. Isolation and characterization of clay-humic complexes. In: *Contaminants and Sediments, Volume 2, Analysis, Chemistry, Biology*. R.A. Baker (ed), Ann Arbor Science, Ann Arbor, pp 207-219.

WERSHAW, R.L., MIKITA, M.A. & STEELINK, C. 1981. Direct ^{13}C NMR evidence for carbohydrate moieties in fulvic acids. *Environmental Science and Technology*, **15**, 1461-1463.

WERSHAW, R.L. & AIKEN, G.R. 1985. Molecular size and weight measurements of humic substances. In: *Humic Substances in Soil, Sediment, and Water: Geochemistry, Isolation, and Characterization*. G.R. Aiken, D.M. McKnight, R.L. Wershaw & P. MacCarthy (eds), Wiley-Interscience, New York, pp 477-492.

WERSHAW, R.L. 1986. A new model for humic materials and their interactions with hydrophobic organic chemicals in soil-water or sediment-water systems. *Journal of Contaminant Hydrology*, **1**, 29-45.

WERSHAW, R.L., THORN, K.A. & PINCKNEY, D.J. 1988. Characterization of humic acid fractions by C-13 nuclear magnetic resonance spectroscopy. *Environmental Technology Letters*, **9**, 53-62.

WHITEHOUSE, B.G. 1985. The effects of dissolved organic matter on the aqueous partitioning of polynuclear aromatic hydrocarbons. *Estuarine Coastal and Shelf Science*, **20**, 393-402.

WHITELEY, L.D. & MARTIN, C.R. 1987. Perfluorosulfonate ionomer film coated electrodes as electrochemical sensors: fundamental investigations. *Analytical Chemistry*, **59**, 1746-1751.

WHITFIELD, M. & TURNER, D.R. 1979. Critical assessment of the relationship between biological, thermodynamic and electrochemical availability. In: *Chemical Modeling in Aqueous Systems*. E.A. Jenne (ed), American Chemical Society Symposium Series 93, Washington, pp 657-680.

WILLIAMS, K.W. 1972. Solute-gel interactions in gel filtration. *Laboratory Practice*, **21**, 667-670.

WILSON, D.E. & KINNEY, P. 1977. Effects of polymeric charge variations on the proton-metal ion equilibria of humic materials. *Limnology and Oceanography*, **22**, 281-289.

WILSON, J.G. 1990. Phenolic analogues of amino carboxylic acid ligands for ^{99m}Tc. III. N-(2-hydroxybenzyl)sarcosines (hbs). *Australian Journal of Chemistry*, **43**, 783-789.

WILSON, M.A. & GOH, K.M. 1977. Proton-decoupled pulse Fourier-transform ^{13}C magnetic resonance of soil organic matter. *Journal of Soil Science*, **28**, 645-652.

WILSON, M.A. 1981. Applications of nuclear magnetic resonance spectroscopy to the study of the structure of soil organic matter. *Journal of Soil Science*, **32**, 167-186.

WILSON, M.A. & GOH, K.M. 1981. Comment on the paper: "[^{1}H] and [^{13}C] NMR studies on the importance of aromatic structures in fulvic and humic acids" (Ruggiero et al., 1979). *Geochimica et Cosmochimica Acta*, **45**, 489-490.

WILSON, M.A., PUGMIRE, R.J. & GRANT, D.M. 1983a. Nuclear magnetic resonance spectroscopy of soils and related materials. Relaxation of ^{13}C nuclei in cross polarization nuclear magnetic resonance experiments. *Organic Geochemistry*, **5**, 121-129.

WILSON, M.A., PHILP, R.P., GILLAM, A.H., GILBERT, T.D. & TATE, K.R. 1983b. Comparison of the structures of humic substances from aquatic and terrestrial sources by pyrolysis gas chromatography-mass spectrometry. *Geochimica et Cosmochimica Acta*, **47**, 497-502.

WILSON, M.A., GOH, K.M., COLLIN, P.J. & GREENFIELD, L.G. 1986. Origins of humus variation. *Organic Geochemistry*, **9**, 225-231.

WILSON, M.A., VASSALLO, A.M., PERDUE, E.M. & REUTER, J.H. 1987. Compositional and solid-state nuclear magnetic resonance study of humic and fulvic acid fractions of soil organic matter. *Analytical Chemistry*, **59**, 551-558.

WILSON, S.A., HUTH, T.C., ARNDT, R.E. & SKOGERBOE, R.K. 1980. Voltammetric methods for determination of metal binding by fulvic acid. *Analytical Chemistry*, **52**, 1515-1518.

WINNER, R.W. 1984. The toxicity and bioaccumulation of cadmium and copper as affected by humic acid. *Aquatic Toxicology*, **5**, 267-274.

WINNER, R.W. 1985. Bioaccumulation and toxicity of copper as affected by interactions between humic acid and water hardness. *Water Research*, **19**, 449-455.

WINNER, R.W. & GAUSS, J.D. 1986. Relationship between chronic toxicity and bioaccumulation of copper, cadmium and zinc as affected by water hardness and humic acid. *Aquatic Toxicology*, **8**, 149-161.

WOODWELL, G.M., CRAIG, P.P. & JOHNSON, H.A. 1971. DDT in the biosphere: where does it go? *Science*, **174**, 1101-1107.

WOOF, J.B. & PIERCE, J.S. 1967. Separation of complex mixtures of polyhydroxy phenols on columns of Sephadex. *Journal of Chromatography*, **28**, 94-103.

WOJCIECHOWSKI, M. & BALCERZAK, J. 1990. Square-wave anodic stripping voltammetry at glassy-carbon-based thin mercury film electrodes in solutions containing dissolved oxygen. *Analytical Chemistry*, **62**, 1325-1331.

WU, Y.C., KOCH, W.F. & MARINENKO, G. 1984. A report on the National Bureau of Standards pH standards. *Journal of Research. National Bureau of Standards*, **89**, 395-400.

XUE, H-B., STUMM, W. & SIGG, L. 1988. The binding of heavy metals to algal surfaces. *Water Research*, **22**, 917-926.

YOUNG, S.D., BACHE, B.W., WELCH, D. & ANDERSON, H.A. 1981. Analysis of the potentiometric titration of natural and synthetic polycarboxylates. *Journal of Soil Science*, **32**, 579-592.

YOUNG, S.D., BACHE, B.W. & LINEHAN, D.J. 1982. The potentiometric measurement of stability constants of soil polycarboxylate-Cu^{2+} chelates. *Journal of Soil Science*, **33**, 467-475.

YOUNG, S.D. & BACHE, B.W. 1985. Aluminium-organic complexation: formation constants and a speciation model for the soil solution. *Journal of Soil Science*, **36**, 261-269.

YUAN, T.L. 1964. Comparison of reagents for soil organic matter extraction and effect of pH on subsequent separation of humic and fulvic acids. *Soil Science*, **98**, 133-141.

ZEPP, R.G., BAUGHMAN, G.L. & SCHLOTZHAUER, P.F. 1981a. Comparison of photochemical behavior of various humic substances in water: I. Sunlight induced reactions of aquatic pollutants photosensitized by humic substances. *Chemosphere*, **10**, 109-117.

ZEPP, R.G., BAUGHMAN, G.L. & SCHLOTZHAUER, P.F. 1981b. Comparison of photochemical behavior of various humic substances in water: II. Photosensitized oxygenations. *Chemosphere*, **10**, 119-126.

ZEPP, R.G., SCHLOTOZHAUER, P.F. & SINK, R.M. 1985. Photosensitized transformations involving electronic energy transfer in natural water. Role of humic substances. *Environmental Science and Technology*, **19**, 74-81.

ZHANG, M. & FLORENCE, T.M. 1987. A novel adsorbent for the determination of the toxic fraction of copper in natural waters. *Analytica Chimica Acta*, **197**, 137-148.

ZHOU, X. & WANGERSKY, P.J. 1989. Study of copper-complexing organic ligands: isolation by a Sep-Pak C18 column extraction technique and characterization by Chromarod thin-layer chromatography. *Marine Chemistry*, **26**, 21-40.